THE BIOCHEMICAL JOURNAL

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The Influence of Structure on the Hydrolysis of Substituted Phenyl β-D-glucosides by Emulsin

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(*Received* 6 *October* 1953)

Detailed information on the mode of interaction of an enzyme with its substrate can probably best be obtained by correlating enzyme activity with structural changes in the substrate. Such information is of interest both in itself and as a basis for chemotherapeutic studies, and the present paper is the first of a series in which it is proposed to study a number of enzyme reactions from this point of view.

The enzyme emulsin was chosen for this initial study since it is comparatively unspecific, catalysing the hydrolysis of a wide range of glycosides. The group of substrates chosen for the work was that of the phenyl β-D-glucosides, in which structural changes can readily be brought about by the introduction of substituents into the benzene ring; the electronic effects of such substituents can be measured quantitatively by means of the substitution constants (σ values) of Hammett (1938, 1940). Following the work of Josephson (1925), the action of emulsin on various glucosides has been extensively studied by Helferich & Veibel and their colleagues (for refs. see Pigman, 1944, and Pigman & Goepp, 1948); this large volume of work has not, however, led to any very clear conclusions, partly because the structural variation in the aglucones has not been sufficiently sytematic and partly because of variations in the experimental conditions, but mainly because, in most cases, the results have been expressed only in terms of the overall velocity constant of the reaction. According to the widely accepted views of Michaelis & Menten (1913), the action of an enzyme on a substrate falls into two distinct stages, namely, reversible combination to form an enzyme-substrate complex and irreversible breakdown of this complex into regenerated enzyme and products; structural changes in the substrate will not necessarily have similar effects on these two stages of the overall reaction and it is therefore desirable to obtain a quantitative measure of both, the first in terms of its equilibrium constant (the reciprocal of the usual Michaelis constant) and the second in terms of its velocity constant.

We describe below the determination of both of these constants for the emulsin-catalysed hydrolysis of phenyl β-D-glucoside and twenty of its substitution products, together with the determination of the velocity constants for the acid- and alkali-catalysed hydrolyses of the same series of glucosides.

EXPERIMENTAL

Preparation of the glucosides

(Light petroleum denotes the fraction of b.p. 60–80°.)

The glucosides were all prepared by way of their tetra-acetyl derivatives, most of which were prepared by the following procedure (cf. Helferich & Schmitz-Hillebrecht, 1933; Sisido, 1936). The phenol (0·3–0·4 mole) was heated with penta-*O*-acetyl-β-D-glucose (0·1 mole; Fischer, 1916) and *p*-toluenesulphonic acid (0·7–2·0 g.) under reduced pressure at 100–120° for 1 hr. 30 min. to 2 hr. The cooled product was dissolved in benzene (200 ml.) and the solution washed with water and then repeatedly with 10% (w/v) NaOH soln. until the washings were colourless. The solution was again washed with water, dried ($CaCl_2$) and evaporated under reduced pressure. The residue was crystallized from methanol using charcoal; this crude product was then crystallized to constant m.p. from a suitable solvent. In certain cases (*o-tert.*-butyl, 2:6-dimethyl and *p*-methoxy) it was found advantageous to run the washed benzene extract through a column of alumina, eluting with more benzene, in order to remove dark, alkali-insoluble impurities.

In four cases (the *o*-nitro- and *o*-, *m*- and *p*-cyano compounds) the method of Robertson & Waters (1930) was used, thus: acetobromoglucose (0·05 mole; Fischer, 1916), the requisite phenol (0·025 mole) and dry, redistilled quinoline (about 50 ml.) were made into a paste with which dry, freshly prepared 'active' silver oxide (0·05 mole; Helferich & Klein, 1926) was thoroughly triturated. The mixture was kept in a desiccator for 1 hr. 30 min., stirring every 15 min., and then extracted with glacial acetic acid (450 ml.) at room temp. and filtered. The filtrate was poured slowly, with mechanical stirring, into water (4 l.) at 0° and the precipitate either collected and directly crystallized or (for the *o*- and *m*-cyano compounds) chromatographed on alumina in benzene/light petroleum, eluted with benzene and crystallized.

The tetra-acetylglucosides were deacetylated (Zemplen & Pacsu, 1929) by dissolving in anhydrous methanol (200–800 ml. for 25 g.) and adding a freshly prepared solution of sodium methoxide (from sodium, 10–20 mg., and methanol, 10 ml.). The mixture was heated on the steam bath for 6–8 min. and then kept at room temp. overnight. The methanol was then removed under reduced pressure and the residual glucoside crystallized to constant m.p. using, if possible, more than one solvent.

The properties of the glucosides used were as follows:

Phenyl β-D-glucoside, crystallized from water, m.p. 171–172° after drying at 130–140° *in vacuo*. (Found: C, 56·5; H, 6·4. Calc. for $C_{12}H_{16}O_6$: C, 56·3; H, 6·25%.) Fischer & Armstrong (1901) give m.p. 174–175° for the anhydrous compound, Robertson & Waters (1930), m.p. 176° for a monohydrate, and Helferich & Schmitz-Hillebrecht (1933), m.p. 144–145° for a dihydrate.

o-Cresyl β-D-glucoside, crystallized from water and ethanol/benzene, m.p. 162–163°; Helferich & Scheiber (1934) give m.p. 163–164°.

m-Cresyl β-D-glucoside, crystallized from water, m.p. 180°; Helferich & Philipp (1934) give m.p. 183–184°.

p-Cresyl β-D-glucoside, crystallized from water and ethanol/benzene, m.p. 179–180°; Helferich & Scheiber (1934) give m.p. 178–179·5°.

o-iso*Propylphenyl* β-D-*glucoside*, was very hygroscopic and resisted all attempts at recrystallization; the product, dried at 100° *in vacuo* over P_2O_5, had m.p. 60–70°, $[\alpha]_D^{21.5}$ −63·8° in water (*c*, 0·6). (Found: C, 60·2; H, 7·5. $C_{15}H_{22}O_6$ requires C, 60·4; H, 7·4%.) The *tetra-acetyl derivative* crystallized from benzene in prismatic needles, m.p. 195–196°, $[\alpha]_D^{22}$ −19·0° in $CHCl_3$ (*c*, 2·05). (Found: C, 59·5; H, 6·8. $C_{23}H_{30}O_{10}$ requires C, 59·2; H, 6·4%.)

p-iso*Propylphenyl* β-D-*glucoside* crystallized from water and ethanol/light petroleum in plates, m.p. 160–161°, $[\alpha]_D^{21}$ −50·2° in ethanol (*c*, 1·28). (Found: C, 59·8; H, 7·6. $C_{15}H_{22}O_6$ requires C, 60·4; H, 7·4%.) The *tetra-acetyl derivative* crystallized from methanol in plates, m.p. 177–179°, $[\alpha]_D^{22}$ −18·2° in $CHCl_3$ (*c*, 1·15). (Found: C, 59·4; H, 6·7. $C_{23}H_{30}O_{10}$ requires C, 59·2; H, 6·4%.)

o-tert.-*Butylphenyl* β-D-*glucoside* was very hygroscopic and resisted all attempts at recrystallization; the product, dried at 100° *in vacuo* over P_2O_5, had m.p. 70–90°, $[\alpha]_D^{23}$ −64·1° in water (*c*, 0·62). (Found: C, 60·5; H, 7·9. $C_{16}H_{24}O_6$ requires C, 61·5; H, 7·7%.) The *tetra-acetyl derivative* crystallized from ethanol in plates, m.p. 191°, $[\alpha]_D^{22}$ −34·8° in $CHCl_3$ (*c*, 1·32). (Found: C, 60·1; H, 6·8. $C_{24}H_{32}O_{10}$ requires C, 60·0; H, 6·7%.)

p-tert.-Butylphenyl β-D-glucoside, crystallized from ether/light petroleum, m.p. 145–146°; Helferich, Scheiber & Hiltmann (1940) give m.p. 148–149·5°.

2:4-Dimethylphenyl β-D-glucoside, crystallized from ethyl acetate/light petroleum and water, m.p. 175–176°, $[\alpha]_D^{21}$ −72·4° in water (*c*, 0·58). (Found: C, 59·0; H, 7·6. Calc. for $C_{14}H_{20}O_6$: C, 59·2; H, 7·05%.) Helferich, Scheiber, Streeck & Vorsatz (1935) give m.p. 181–183·5°, $[\alpha]_D^{20}$ −65°. The tetra-acetyl derivative, crystallized from ethanol/light petroleum, had m.p. 122–123°, $[\alpha]_D^{22}$ −18·8° in $CHCl_3$ (*c*, 1·6). (Found: C, 58·6; H, 6·3. Calc. for $C_{22}H_{28}O_{10}$: C, 58·4; H, 6·2%.) Helferich *et al.* (1935) give m.p. 171–173°, which appears to be in error, and $[\alpha]_D$ −20°.

2:6-Dimethylphenyl β-D-glucoside, crystallized from water, m.p. 209–211°; Helferich & Scheiber (1934) give m.p. 209–211°.

o-Methoxyphenyl β-D-glucoside, crystallized from water as the monohydrate, m.p. 155–156° (Helferich & Burt, 1935, give m.p. 155·5–157·5°), which became anhydrous after 2 hr. *in vacuo* at 110–120°. (Found: C, 54·1; H, 6·5. Calc. for $C_{13}H_{18}O_7$: C, 54·5; H, 6·3%.)

m-*Methoxyphenyl* β-D-*glucoside*, crystallized from water and ethanol/light petroleum in needles, m.p. 159–161°, $[\alpha]_D^{18}$ −69·2° in water (*c*, 1·04). (Found: C, 54·9; H, 6·45. $C_{13}H_{18}O_7$ requires C, 54·5; H, 6·3%.) The *tetra-acetyl derivative* crystallized from ethanol in plates, m.p. 130–131°, $[\alpha]_D^{15}$ −20·6° in $CHCl_3$ (*c*, 3·2). (Found: C, 55·8; H, 5·95. $C_{21}H_{26}O_{11}$ requires C, 55·5; H, 5·7%.)

p-Methoxyphenyl β-D-glucoside, crystallized from water, m.p. 175–177°; Helferich & Schmitz-Hillebrecht (1933) give m.p. 175–177°.

o-*Chlorophenyl* β-D-*glucoside*, crystallized from water and ethanol in needles, m.p. 170–171°, $[\alpha]_D^{18.5}$ −90·6° in water (*c*, 0·73). (Found: C, 49·7; H, 5·4. $C_{12}H_{15}O_6Cl$ requires C, 49·6; H, 5·2%.) The *tetra-acetyl derivative* crystallized from benzene/light petroleum in prisms, m.p. 149–150°, $[\alpha]_D^{16}$ −51·6° in $CHCl_3$ (*c*, 1·45). (Found: C, 51·65; H, 5·2. $C_{20}H_{23}O_{10}Cl$ requires C, 52·4; H, 5·0%.)

m-*Chlorophenyl* β-D-*glucoside*, crystallized from ethanol, ethyl acetate and water in prismatic needles, m.p. 179–180°, $[\alpha]_D^{16}$ −36·3° in water (*c*, 0·59). (Found: C, 49·8; H, 5·4. $C_{12}H_{15}O_6Cl$ requires C, 49·6; H, 5·2%.) The *tetra-acetyl derivative* crystallized from methanol in needles, m.p. 131·5–133·5°, $[\alpha]_D^{16}$ −22·8° in $CHCl_3$ (*c*, 1·79). (Found: C, 52·4; H, 5·1. $C_{20}H_{23}O_{10}Cl$ requires C, 52·4; H, 5·0%.)

p-Chlorophenyl β-D-glucoside, crystallized from water, m.p. 173–175°, $[\alpha]_D^{19}$ −82·0° in water (*c*, 1·13). (Found: C, 49·6; H, 5·4. Calc. for $C_{12}H_{15}O_6Cl$: C, 49·6; H, 5·2%.) Dyfverman & Lindberg (1950) give m.p. 173–174°, $[\alpha]_D^{20}$ −69·5°.

o-*Cyanophenyl* β-D-*glucoside* crystallized from ethyl acetate/light petroleum in prisms, m.p. 176–177°, $[\alpha]_D^{18.5}$ −70·9° in water (*c*, 1·55). (Found: C, 55·6; H, 5·6. $C_{13}H_{15}O_6N$ requires C, 55·5; H, 5·35%.) The *tetra-acetyl derivative* crystallized from ethanol, m.p. 163–164°, $[\alpha]_D^{18}$ −14·2° in $CHCl_3$ (*c*, 2·28). (Found: C, 56·2; H, 5·25; N, 3·1. $C_{21}H_{23}O_{10}N$ requires C, 56·1; H, 5·1; N, 3·1%.)

m-*Cyanophenyl* β-D-*glucoside*, crystallized from water, ethyl acetate/light petroleum, ethanol/light petroleum and ethanol in fine prisms, m.p. 167–168°, $[\alpha]_D^{18.5}$ −137·7° in water (*c*, 1·59). (Found: C, 56·2; H, 5·6. $C_{13}H_{15}O_6N$ requires C, 55·5; H, 5·35%.) The *tetra-acetyl derivative* crystallized from ethanol in prismatic needles, m.p. 126–127°, $[\alpha]_D^{18}$ −31·5° in $CHCl_3$ (*c*, 2·34). (Found: C, 56·45; H, 5·3. $C_{21}H_{23}O_{10}N$ requires C, 56·1; H, 5·1%.)

p-*Cyanophenyl* β-D-*glucoside*, crystallized from water in long prisms, m.p. 193–194°, $[\alpha]_D^{18.5}$ −91·9° in water (*c*, 1·51). (Found: C, 55·6; H, 5·6. $C_{13}H_{15}O_6N$ requires C, 55·5; H, 5·35%.) The *tetra-acetyl derivative* crystallized from methanol in long prisms, m.p. 151–153°, $[\alpha]_D^{18.5}$ −29·4° in $CHCl_3$ (*c*, 2·90). (Found: C, 56·1; H, 5·2; N, 3·35. $C_{21}H_{23}O_{10}N$ requires C, 56·1; H, 5·1; N, 3·1%.)

o-Nitrophenyl β-D-glucoside crystallized from water as a hydrate, which lost its water at 110° over P_2O_5 *in vacuo* and then had m.p. 168–170°, $[\alpha]_D^{18.5}$ −140·4° in water (*c*, 2·14). (Found: C, 48·2; H, 5·25. Calc. for $C_{12}H_{15}O_8N$: C, 47·8; H, 5·0%.) Montgomery, Richtmyer & Hudson (1942) give m.p. 152°, $[\alpha]_D$ −106°, for the anhydrous compound, while Glaser & Wulwek (1924) give m.p. 130–131°, $[\alpha]_D^{14}$ −82·7°, for the hydrate.

m-Nitrophenyl β-D-glucoside, crystallized from water and dried *in vacuo* at 110°, had m.p. 166–168°, $[\alpha]_D^{18.5}$ −87·3° in water (*c*, 1·92). (Found: C, 47·4; H, 5·2. Calc. for $C_{12}H_{15}O_8N$: C, 47·8; H, 5·0%.) Glaser & Wulwek (1924) give m.p. 167–168°, $[\alpha]_D^{22}$ −84·9°, for the monohydrate.

p-Nitrophenyl β-D-glucoside, crystallized from water, m.p. 164–165°; Glaser & Wulwek (1924) give m.p. 164–165°.

Analytical methods

In most previous work on the hydrolysis of glucosides by emulsin the reaction was followed polarimetrically, a sample of the reaction mixture being withdrawn at an appropriate time, added to saturated K_2CO_3 solution, allowed to mutarotate and examined in the polarimeter. Although simple, this method suffers, as Veibel & Eriksen (1936) have pointed out, from serious disadvantages; thus, allowance has to be made for the initial rotation of the reaction mixture and this is determined at the reaction pH (4·4–5·0), whereas the final rotation is determined at pH 10·5–10·6, at which the specific rotations of glucose and the glucosides differ significantly from those at the lower pH; moreover, appreciable decomposition of glucose occurs during the period allowed for mutarotation. Although suitable corrections can be made, we have preferred to determine either the glucose or the phenol produced by the hydrolysis by direct chemical methods, thus obviating these uncertainties.

The enzymic hydrolyses of all but the cyano- and nitrophenyl glucosides were followed by estimating the glucose formed using a modification of the method of Jendrassik & Polgar (1940) (cf. Fujita & Iwatake, 1931). The procedure was as follows (the various solutions are those described by Jendrassik & Polgar, 1940): 1 ml. of the glucose solution was mixed with 0·8 ml. of 0·1 N-NaOH and 0·5 ml. of cadmium sulphate solution. The mixture was heated in a boiling-water bath for 3 min. and filtered through a plug of washed cotton wool, which was then thoroughly washed with hot water. To the filtrate and washings, 2 ml. of alkaline ferricyanide solution were added and the mixture was heated in a boiling-water bath for 15 min., cooled and titrated with 0·005 N-$Na_2S_2O_3$, after adding 0·5 ml. of acid $ZnSO_4$ solution and a few drops of starch-iodide solution. A blank was carried out simultaneously using 1 ml. of 0·005 M acetate buffer, pH 5·25, in place of the glucose solution. If a (ml.) is the titre of the glucose solution and b that of the blank, the molar concentration of glucose is $0{\cdot}000965\, f(b-a)$, where f is the factor of the $Na_2S_2O_3$. In twenty control experiments, using standard glucose solutions, the error fell between +1·0% and −3·0%. The same method, except that the cadmium sulphate treatment was omitted and the solution was neutralized with the calculated amount of 0·01 N-NaOH before adding the alkaline ferricyanide, was used for following the acid-catalysed hydrolyses of the same glucosides; the error in the control experiments fell within the limits +0% and +3·5%.

This method failed for the cyano compounds, and the enzyme- and acid-catalysed hydrolyses of the *m*- and *p*-cyanophenyl glucosides were followed by estimating the liberated glucose in 1 ml. samples by the method of Folin & Wu (1920) (cf. Hawk, Oser & Summerson, 1947*a*). The final colour was measured in a 1 cm. cell, against a water blank, with a Spekker Photoelectric Absorptiometer using an Ilford Spectrum Red filter, no. 608 (wavelength of peak transmission, 680 mμ.); a standard curve covering the range up to 9 mM glucose (Spekker reading, 0·65) was used. The method was unsatisfactory for the *o*-cyanophenyl glucoside.

The alkali-catalysed hydrolyses of all but the nitrophenyl glucosides were followed by estimation of the liberated phenols by the method of Folin & Ciocalteu (1927) (cf. Hawk, Oser & Summerson, 1947*b*); methods based on the estimation of glucose cannot be used, owing to the instability of glucose in alkaline solution, while the polarimetric method employed by Dyfverman & Lindberg (1950) was not applicable to our more dilute solutions. The final colour was measured in a 1 cm. cell with a Spekker Photoelectric Absorptiometer, using an Ilford Spectrum Red filter, no. 608 (680 mμ.); reference curves were constructed for each phenol covering the range up to 1·4 mM (Spekker reading, 0·3–0·9).

The nitrophenols were estimated in enzymic, acid and alkaline hydrolysates by means of their yellow colour in alkaline solution. The reaction mixture (1 ml.) was neutralized if necessary and so diluted with water and NaOH solution as to give a final NaOH concentration of 0·01 N for the *o*- and *p*-compounds and 0·033 N for the *m*-compound. The yellow colour was measured in a 1 cm. cell with a Spekker photoelectric absorptiometer using an Ilford Spectrum Violet filter, no. 601 (wavelength of peak transmission, 430 mμ.). Reference curves were made for each phenol, covering the range up to 16×10^{-5} M (Spekker readings, 0·194, 0·210 and 0·655 for *o*-, *m*- and *p*-nitrophenols respectively).

Preparation of emulsin

The enzyme was prepared from sweet almond meal by the tannin precipitation method of Helferich, Winkler, Gootz, Peters & Günther (1932); the alcohol-extraction procedure of Tauber (1932) gave a much less active product. The product (the 'Rohferment' of Helferich *et al.* 1932) was washed free of tannin (no blue colour with $FeCl_3$) with acetone and dried over H_2SO_4 *in vacuo* at 0° for 3 days. After equilibration with atmospheric moisture it was preserved at 0° over silica gel. The yield of material containing 12·1% nitrogen was 1·4%, based on the sweet almond meal used.

β-D-Glucosidase activity is conveniently expressed as the Ph.f. value (cf. the Sal.f. value used by Josephson, 1925, Weidenhagen, 1929, and Helferich, Gootz & Sparmberg, 1932) which we define as the first-order velocity constant ($min.^{-1}$) for the hydrolysis of phenyl β-D-glucoside in 0·05 M concentration at 30° and pH 5·25 calculated for a standard enzyme concentration of 100 mg./l. (the reaction was shown experimentally to be first order with respect to the enzyme). The Ph.f. value of the sample used for all our work changed negligibly during the course of the work, being 4·44 at the beginning (March, 1950) and 4·28 at the end (June, 1951). A Ph.f. value of 4·44 corresponds to an 'enzyme value' ('Wertigkeit') (Helferich, 1938) of 0·30 and may be compared with Helferich's value of 0·33 for his preparation of 'Rohferment.'

A small amount of the standard emulsin was further purified by the silver precipitation method of Helferich & Winkler (1932); the enzyme from the second precipitate

Table 1. *Comparison of equilibrium constants using 'Rohferment' and silver-purified enzyme*

	Equilibrium constant, K_a	
β-D-Glucoside	'Rohferment'	Silver-purified enzyme
Phenyl	36·75	32·25
o-Cresyl	43·4	50·75
m-Cresyl	52·95	42·4
p-Cresyl	76·9	71·95

(2·3% yield) had Ph.f. = 20·8 and that from the third precipitate (0·16% yield) Ph.f. = 26·0. As shown in Table 1, this further purification had no very great effect on the equilibrium constants and, owing to the very poor yield of the silver-purified enzyme, we therefore used our 'Rohferment' preparation for enzymic hydrolysis, the same sample being used throughout.

Optimum conditions for enzymic hydrolysis

Veibel & Lillelund (1940*b*) studied the effect of pH on the hydrolysis of a number of glucosides with emulsin and found that the overall velocity constant was maximal at pH 5·0 for *o*-cresyl β-D-glucoside and at pH 4·4 for *n*-butyl β-D-glucoside; they further found that, for *o*-cresyl β-D-glucoside, the equilibrium constant was greatest at pH 5·0–5·2. We determined the overall velocity constant for the hydrolysis of nine of our glucosides at pH 4·5 and 5·0 (see Table 2)

Table 2. *Velocity constants* ($10^4 k_0$; *min.*$^{-1}$) *for hydrolysis of* β-D-*glucosides with emulsin* (100 *mg./l.*) *at* 30°

Substrate concentration, 0·01 M; 0·01 M acetate buffer.

β-D-Glucoside	pH 4·5	pH 5·0
Phenyl	4·34*	4·42*
o-Cresyl	321	342
m-Cresyl	20·5	23·4
o-Methoxyphenyl	322	431
m-Methoxyphenyl	37·1	44·5
p-Methoxyphenyl	23·8	29·1
o-Chlorophenyl	487	643
m-Chlorophenyl	144·5	176·5
p-Chlorophenyl	24·3	27·0

* 0·05 M substrate.

Table 3. *Hydrolysis of phenyl* β-D-*glucoside by emulsin at* 30° *in* 0·005 M *acetate buffer*, pH 5·25

e_0' = Enzyme concentration, mg./l.
s_0 = Substrate concentration, moles/l.
t = Time, min.
a = $Na_2S_2O_3$ titre of 1 ml. of reaction mixture, ml.
b = $Na_2S_2O_3$ titre of 1 ml. enzyme blank, ml.
f = Factor of 0·005 N-$Na_2S_2O_3$.
x = Glucose concentration, moles/l.
k_0 = Overall velocity constant, min.$^{-1}$.
k_3' = First-order velocity constant for decomposition of enzyme-substrate complex, min.$^{-1}$.
K_a = Equilibrium constant for formation of enzyme-substrate complex, l./mole.

Expt. no.	e_0'	s_0	f	b	t	a	$10^4 x$	$10^4 k_0$	$10^3 k_3'$	K_a
46	100	0·008	0·779	2·48	30	2·26	1·65			
					60	2·06	3·16	6·37	24·2	
					90	1·89	4·44			
47	100	0·016	0·779	2·48	30	2·11	2·78			
					60	1·78	5·26	5·41	24·8	33·3
					90	1·47	7·60			
48	100	0·024	0·779	2·48	30	2·03	3·39			
					60	1·60	6·62	4·55	24·6	
					90	1·20	9·63			
49	75	0·006	0·779	2·48	45	2·27	1·58			
					90	2·09	2·93	5·10	21·8	
					135	1·95	3·98			
50	75	0·008	0·779	2·48	45	2·21	2·03			
					90	1·99	3·69	4·81	21·8	
					135	1·81	5·04			38·5
51	75	0·012	0·779	2·48	45	2·14	2·56			
					90	1·83	4·89	4·35	22·0	
					135	1·57	6·84			
52	75	0·016	0·779	2·48	45	2·03	3·39			
					90	1·73	5·64	3·86	21·6	
					135	1·38	8·27			
53	42·5	0·008	0·774	2·48	80	2·25	1·72			
					160	2·05	3·21	2·70	21·6	
					240	1·80	5·08			
54	42·5	0·012	0·774	2·48	80	2·17	2·32			
					160	1·88	4·48	2·40	21·5	38·5
					240	1·58	6·73			
55	42·5	0·016	0·774	2·48	80	2·11	2·76			
					160	1·73	5·61	2·18	21·5	
					240	1·40	8·07			
							Mean values		22·5	36·75
							S.E.		±0·4	±1·7

and found that, in every case, the reaction was faster at pH 5·0; further experiments with five glucosides showed that the velocity constant was almost unaffected by change of pH over the range 5·0–5·5. We therefore adopted pH 5·25 for our work.

Veibel & Lillelund (1940*b*) also showed that acetate buffer had some inhibiting effect on the hydrolysis of *o*-cresyl β-D-glucoside; we therefore chose a low buffer concentration, namely 0·005 M.

All our enzymic hydrolyses were carried out at 30°±0·05° and, in order to avoid the necessity for correcting for interference by the reaction products (cf. Veibel & Eriksen, 1934–6), the enzyme and substrate concentrations were so chosen that the degree of hydrolysis was never more than 20% (in 90% of the experiments it was less than 10%).

Enzymic hydrolyses

The hydrolyses were carried out in 25 ml. stoppered flasks into which were introduced suitable volumes of acetate buffer (prepared according to Green, 1933, and checked by means of a Cambridge pH meter), glucoside solution, water and enzyme solution to give a total volume of 10 ml., with a buffer concentration of 0·005 M and the desired final concentrations of glucoside and enzyme; the enzyme solution, prepared by dissolving a weighed amount in a little water, filtering from a small amount of insoluble material and making up to volume with water, was added last, after preheating the other components in the thermostat for 10 min. At suitable intervals, 1 ml. samples were withdrawn for analysis; in all cases a blank determination was made using the same concentration of enzyme in 0·005 M acetate buffer, pH 5·25; some of the substrates (*m*-cresyl, *o*- and *m*-methoxyphenyl, *o*-chlorophenyl and *p*-cyanophenyl β-D-glucosides) contained small amounts of reducing impurity for which the requisite correction was made.

The overall velocity constant, k_0, was calculated in the usual way from the slope of the linear plot (drawn by the method of least squares) of the decadic logarithm of the concentration of unhydrolysed substrate against the time in minutes. For the determination of K_m, K_a and k_2' (see below) two or three sets of three or four such determinations of k_0, using different enzyme concentrations, were carried out with each substrate.

The complete data for the enzymic hydrolyses of phenyl β-D-glucoside are given in Table 3; the full data for the other twenty glucosides are given by Nath (1951).

Acid and alkaline hydrolyses

The hydrolyses were carried out in 50 ml. flasks fitted with cold-finger condensers (Braude, Jones & Stern, 1946) in an oil thermostat at 60°±0·05°. For the acid hydrolyses the glucosides were taken in 0·2 M concentration in 0·1 N-HCl; for the alkaline hydrolyses 0·01 M solutions in 4 N-NaOH were used. Samples were withdrawn at suitable times, and after neutralization, were analysed by the appropriate method. The first-order velocity constants, k_{acid} and $k_{alk.}$, were evaluated in the usual manner from the satisfactorily linear log plots and are recorded in Table 4. The values of $k_{alk.}$ for the *o*- and *p*-nitrophenyl β-D-glucosides were too high for accurate measurement, the former compound being completely hydrolysed in 1 min. and the latter 80% hydrolysed in 3 min.

RESULTS

The results of the experiments on enzymic hydrolysis are expressed in terms of the equilibrium constant, $K_a\ (=k_1/k_2)$, for the formation of the enzyme-substrate complex and of the first-order velocity constant, k_3, for the decomposition of this complex according to the well-known Michaelis-Menten representation:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E+P.$$

It is more usual to use the Michaelis constant, K_m, which is the dissociation constant for the breakdown of the enzyme-substrate complex into its original components, but we prefer K_a as being more logical, more immediately informative, since it increases with increasing tendency for the overall reaction to proceed from left to right, and more in keeping with standard physico-chemical usage.

Being merely the reciprocal of the Michaelis constant, K_a can be evaluated by any of the methods commonly in use for the evaluation of K_m. We have used the method of Veibel & Lillelund (1940*a*) (cf. Pigman & Goepp, 1948), in which the reciprocal of the overall velocity constant, k_0, at a constant enzyme concentration, is plotted against the substrate concentration; the intercept of the resulting straight line on the x axis is equal to $-K_m = -1/K_a$. This method is preferable to other methods (Michaelis & Menten, 1913; Lineweaver & Burk, 1934; Delory & King, 1943) since it employs k_0, which is accurately determinable, rather than the initial velocity of reaction, which can only be

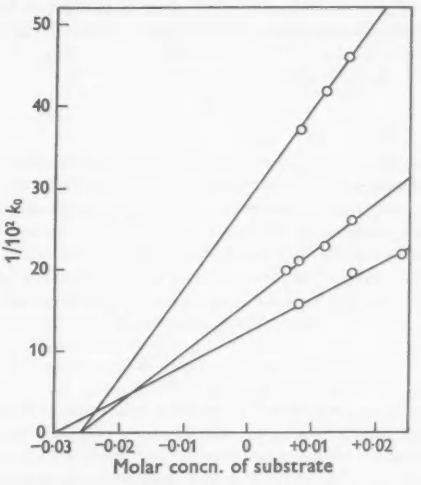


Fig. 1. Determination of K_a for phenyl β-D-glucoside.

Table 4. *Hydrolysis of substituted phenyl β-D-glucosides*

K_a = Equilibrium constant for hydrolysis by emulsin at 30° in 0·005 M acetate buffer, pH 5·25; l./mole.
k_3' = First-order velocity constant for decomposition of enzyme-substrate complex under above conditions; min.$^{-1}$.
k_{acid} = First-order velocity constant for hydrolysis at 60° in 0·02 M solution in 0·1 N-HCl; min.$^{-1}$.
$k_{alk.}$ = First-order velocity constant for hydrolysis at 60° in 0·01 M solution in 4 N-NaOH; min.$^{-1}$.
σ = Substituent constant. (Values for *m*- and *p*-substituents from Hammett, 1940; for *o*-substituents, slightly modified in the light of more recent work, from Mamalis & Rydon, 1950.)

	K_a			$10^3 k_3'$					
Substituent	Mean	± S.E.	No. of determinations	Mean	± S.E.	No. of determinations	$10^4 k_{acid}$	$10^4 k_{alk.}$	σ
H	36·75	± 1·7	3	22·5	± 0·4	10	1·15	2·54	0·000
o-Me	43·4	± 0·5	2	904	± 5	6	1·81	1·16	−0·080
m-Me	52·95	± 4·2	2	56·75	± 1·85	6	1·405	1·92	−0·069
p-Me	76·9	± 0·0	2	17·1	± 0·1	6	2·32	0·960	−0·170
o-Pr^i	345	±12	2	56·05	± 1·8	6	—	0·512	—
p-Pr^i	238	±56	2	9·11	± 0·39	6	—	0·750	−0·151
o-Bu^t	132	± 3·5	2	6·675	± 0·22	6	—	0·137	—
p-Bu^t	157	± 3	2	3·62	± 0·15	6	—	0·672	−0·197
2:4-Me$_2$	171	±11	2	24·9	± 0·3	6	—	0·554	−0·250
2:6-Me$_2$	28·8	± 0·2	2	13·3	± 0·3	6	—	1·152	−0·160
o-MeO	58·1	± 0·55	2	965·5	±10·5	6	8·96	7·62	−0·120
m-MeO	38·5	± 0·75	2	132	± 6	6	0·770	6·28	+0·115
p-MeO	53·45	± 0·85	4	58·8	± 0·95	12	2·407	0·944	−0·268
o-Cl	222	±22	2	832	±27·5	6	1·73	230·3	+0·325
m-Cl	102·5	± 2·5	2	228	± 8	6	0·760	19·7	+0·373
p-Cl	119·5	± 5·5	2	44·0	± 0·75	6	0·830	8·96	+0·227
m-CN	182	± 3·5	2	690·5	±12·5	6	0·509	—	+0·678
p-CN	419·5	±35	2	286	± 3·5	6	0·366	—	+1·000
o-NO$_2$	161·5	± 2·5	2	3515	±29	6	1·66	>50 000	+0·650
m-NO$_2$	222·5	± 5	2	460	±13·5	6	0·404	147·4	+0·710
p-NO$_2$	500	± 0	2	385	± 2	6	0·330	*c.* 5000	+1·270

approximated, and does not require the use of such high substrate concentrations. At least two determinations of K_a were carried out, using different enzyme concentrations, with each substrate; our results for phenyl β-D-glucoside (cf. Table 3) are plotted in Fig. 1.

Veibel & Eriksen (1936–7) and Veibel & Lillelund (1940*a*) showed that, under conditions such that the influence of the products on the velocity of the reaction is negligible,

$$k_3 = \frac{k_0(K_m + s_0)}{e_0}, \qquad (1)$$

where s_0 and e_0 are the initial molar concentrations of substrate and enzyme, respectively. The value of e_0, required for substitution in (1) for the evaluation of k_3, cannot be readily determined since neither the molecular weight of the enzyme nor the purity of the enzyme preparation used are known. We have accordingly replaced e_0 by the weight concentration, e_0' (mg./l.); the resulting expression

$$k_3' = k_0 \frac{(K_m + s_0)}{e_0'} \qquad (2)$$

gives k_3', which is the required first-order constant, k_3, multiplied by a constant factor (assuming our emulsin preparation to be 1 % pure and a molecular weight of 100 000 for pure emulsin, this factor would be 1×10^{-10}). For our present purpose of correlation with substrate structure, k_3' is just as useful as k_3. On rearrangement, (2) takes the form (3),

$$\frac{1}{k_0} = \frac{1}{k_3' e_0'}(K_m + s_0), \qquad (3)$$

which is the equation for the linear plot of $1/k_0$ against s_0, which was used in evaluating K_a; clearly k_3' could be determined from the slope of this line but we have preferred the arithmetical method as being more accurate and very nearly as easy.

The values of K_a and k_3' and of the first-order velocity constants, k_{acid} and $k_{alk.}$, for acid and alkaline hydrolysis of the glucosides investigated by us are given in Table 4.

DISCUSSION

The logarithms of the equilibrium constants, K_a, are plotted against the substituent constants, σ, in Fig. 2. With the exception of the *p-iso*propyl (*p*-Pr^i) and *p-tert.*-butyl (*p*-Bu^t) compounds, the fit is about as good as is usually observed in such correlations. The plot clearly shows that the formation of the enzyme-substrate complex is facilitated by the presence of electron-attracting substituents in the phenyl group. The fact that the points for the *o*-substituents fall satisfactorily on the line indicates that steric effects play a negligible part in the interaction or, at any rate, that part of it directly

influenced by electron-displacement effects; it follows that this interaction must take place in a part of the molecule remote from the phenyl group, namely in the glucose moiety. The most likely form of such interaction subject to polar influences is hydrogen bonding and the facilitating influence of electron-attracting substituents indicates that this must involve the hydroxyl hydrogens of the glucose residue and hydrogen-accepting groups in the enzyme molecule; the alternative possibility of hydrogen bonding between the oxygens of the glucose residue and hydrogens of the enzyme is excluded, since this would be hindered by electron-attracting substituents in the phenyl groups. The observations of Helferich & Lang (1933) and of Pigman & Richtmyer (1942), that certain *O*-methylated glucosides are not hydrolysed by emulsin, are in accordance with the suggested mechanism.

The marked facilitation of enzyme-substrate combination by the bulky alkyl groups, *o*- and *p*-*iso*propyl and *tert.*-butyl, in spite of their electron-repelling properties, suggests that there is some other type of interaction, probably by means of van der Waals forces, involving the aryl group. That the aglycone half of the molecule must be involved follows from the specificity of the enzyme for β-glucosides and the suggestion that it is attached by van der Waals forces is in accord with the observation of Veibel & Lillelund (1938) that the equilibrium constant for combination with alkyl β-D-glucosides increases with increasing size of the alkyl group.

The stereochemical specificity of emulsin renders it likely that at least three centres are involved in the attachment of the substrate to the enzyme (Rydon, 1948) and the following partial representation is advanced as at least a plausible working hypothesis:

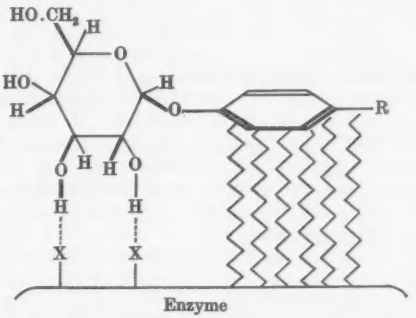


Fig. 3. Possible mode of attachment of a substituted phenyl β-D-glucoside to emulsin. (The glucopyranose ring lies in the plane of the paper, the benzene ring at right angles to it. —H···X—, hydrogen bond; ∿∿∿, van der Waals interaction.)

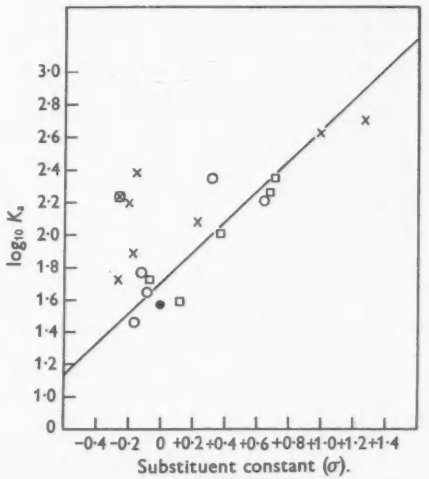
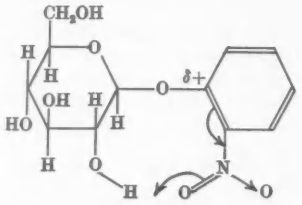


Fig. 2. Correlation of K_a with structure. ●, phenyl β-D-glucoside; ○, *o*-substituted phenyl β-D-glucosides; □, *m*-substituted phenyl β-D-glucosides; ×, *p*-substituted phenyl β-D-glucosides. The line was drawn by the least-square method, neglecting the points for the *o*-substituted compound and for the *p-iso*propyl and *p-tert.*-butyl compounds.

In the figure, the two hydrogen-bonds leading to the glucose residue are intended to represent, but not specify, at least two bonds of this type between the enzyme and the glucopyranose ring.

The logarithms of the first-order velocity constants, $k_{alk.}$ and k_{acid}, for the alkali- and acid-catalysed hydrolyses are plotted against σ in Figs. 4 and 5. The effect of substitution on the two reactions is that expected on general grounds, the acid-catalysed reaction, which depends on attack by the electrophilic proton, being facilitated by electron-repelling substituents, and the alkali-catalysed reaction, in which the attack is by the nucleophilic hydroxyl ion, being facilitated by electron-attracting groups. The reaction constants, ρ (Hammett, 1938, 1940) for the two reactions, $+2{\cdot}48$ and $-0{\cdot}66$, respectively, are normal for the two types of reaction (cf. Taft, 1952); Dyfverman & Lindberg (1950) found $\rho = 2{\cdot}5$ in an investigation of the alkaline hydrolysis of a less extensive series of substituted phenyl β-D-glucosides. Both reactions show facilitation by *o*-nitro, *o*-chloro and *o*-methoxy groups. Facilitation of alkaline hydrolysis by an *o*-nitro group is very pronounced and has been noted by previous workers (Montgomery, Richtmyer & Hudson, 1943; Snyder & Link, 1952); it is readily explicable on the basis of hydrogen bonding with the 2-hydroxyl group of the glucose residue,

which leads to enhanced electrophilic reactivity of the relevant carbon atom in the phenyl group, thus:



and the weaker facilitation by *o*-chloro and *o*-methoxy groups may have a similar explanation. The effect of these three substituents on the acid-catalysed hydrolysis is not readily explicable. The bulky *iso*propyl and *tert.*-butyl groups, when present as *o*-substituents, cause marked inhibition of alkaline hydrolysis, no doubt owing to conventional steric hindrance.

Fig. 6 shows log k'_3 plotted against σ. It is at once apparent that the decomposition of the enzyme-substrate complex, although it occurs in acid solution, resembles alkaline, rather than acid, hydrolysis (contrast Pigman & Goepp, 1948) in being facilitated by electron-attracting substituents. Clearly, the enzyme-substrate complex decomposes under the attack of some nucleophilic reagent; whether this is free hydroxyl ion, adsorbed hydroxyl ion or hydroxyl ion arising from hydrated amino-groups, is a question which cannot profitably be discussed on the basis of the available evidence.

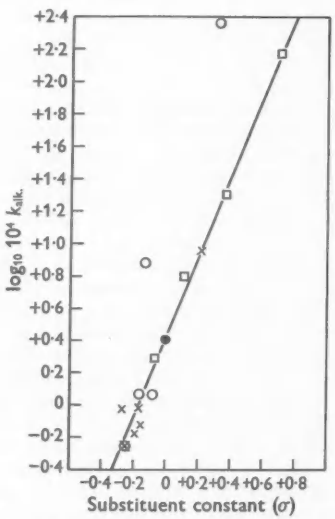


Fig. 4. Correlation of $k_{alk.}$ with structure. For key, see Fig. 2. The line was drawn by the least-square method, neglecting the points for the *o*-substituted compounds.

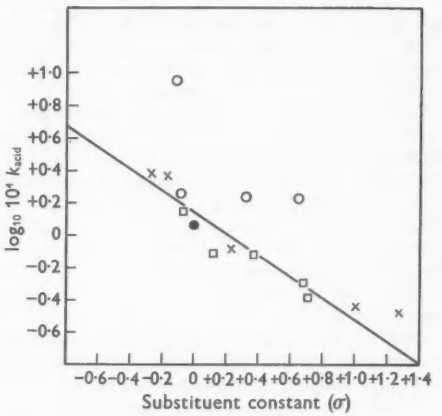


Fig. 5. Correlation of k_{acid} with structure. For key, see Fig. 2. The line was drawn by the least-square method, neglecting the points for the *o*-substituted compound.

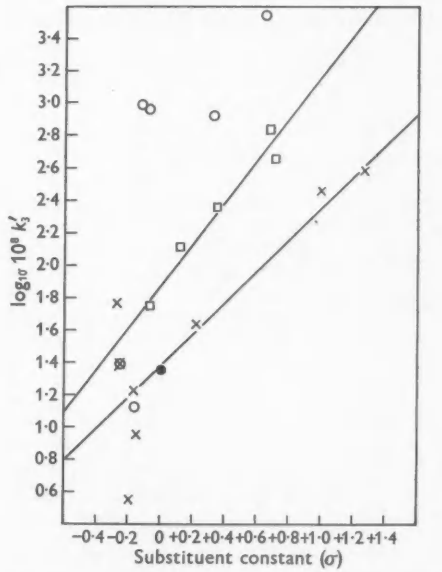


Fig. 6. Correlation of k'_3 with structure. For key, see Fig. 2. The lines were drawn by the least-square method, the upper one using the points for the *m*-substituted compounds and the lower one using the points for the unsubstituted and *p*-substituted compounds.

It is also clear from Fig. 6 that marked steric influences are superimposed on the more usual electronic influences. The points for the *p*-substituted compounds fall reasonably well on a straight line passing through the point for the unsubstituted compound, with the exception of those for the *p-iso*propyl and *p-tert.*-butyl compounds; the sluggish decomposition of these two complexes is very probably due to the strength of the van der Waals component of the forces holding them together, and it may well be that it is the enzyme-product complex which is unusually stable in these cases. The *m*-substituted compounds also fall well on a straight line which is, however, considerably displaced towards higher k_3' values from the point for the unsubstituted compound. The *o*-substituted compounds, again with the exception of the *iso*propyl and *tert.*-butyl compounds, which, like their *p*-isomerides, may owe their stability to strong van der Waals interaction, and also with the curious exception of the dimethyl compounds, show very markedly enhanced instability; the ratios of the values of k_3' for the *o*- and *p*-isomerides are as follows:

Substituent	Me	Cl	MeO	NO_2	Pr^i	Bu^t
k_3' for *o*-/k_3' for *p*-	52·8	18·9	16·3	9·1	6·2	1·8

Clearly the effect here, and also in the *m*-series, is due to the size, rather than the electronic properties, of the substituent, and it is tempting to think that what is involved is a tilting of the phenyl group in Fig. 3, aided by the close fitting of the substituent into a recess in the enzyme of about the size and shape of a methyl group, which results in the key carbon atom of the phenyl group becoming more accessible to attack by the nucleophilic entity involved; more experimental evidence is, however, required before this matter can be pursued further.

SUMMARY

1. The hydrolysis by emulsin of phenyl β-D-glucoside and twenty of its substitution products has been studied; the equilibrium constants for the formation of the enzyme-substrate complexes and the first-order velocity constants for their decomposition have been evaluated.

2. First-order velocity constants for the alkaline and acid hydrolysis of most of the glucosides have also been determined.

3. The results have been correlated with the electronic properties of the substituents, as measured by their substituent constants; acid hydrolysis is facilitated by electron-repelling substituents but alkaline hydrolysis and the formation and breakdown of the enzyme-substrate complex are all facilitated by electron-attracting substituents.

4. A mechanism is suggested for the combination of emulsin with aryl β-D-glucosides.

5. The mechanism of the decomposition of the enzyme-substrate complex is discussed; in this reaction steric effects, as well as electronic effects, appear to play an important part.

We express our grateful thanks to the governments of India and Assam for a scholarship which enabled one of us (R.L.N.) to take part in this work, to the University of London for a grant from the Central Research Fund, to Messrs Stafford, Allen and Sons Ltd. for a gift of sweet almond meal, and to Imperial Chemical Industries Ltd. for the gift of some of the phenols used. Micro-analyses were carried out in the micro-analytical laboratories of this Department (Mr F. H. Oliver).

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The Need for Selenite and Molybdate in the Formation of Formic Dehydrogenase by Members of the *Coli-aerogenes* Group of Bacteria

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Factors required in trace amounts for growth are generally considered to play a catalytic role in the metabolism of the organism, and comparison of the enzymic activity of normal and deficient cells has yielded information concerning the function of organic growth factors in the metabolism of micro-organisms. Waring & Werkman (1944) have used this method to study the function of iron in *Aerobacter indologenes*; they found that the activity of formic dehydrogenase, among other enzymes, was greatly reduced in iron-deficient cells. It does not follow, however, that only such inorganic factors as are required for normal growth are concerned in the formation of enzymes, since an enzyme whose function is not indispensable to the anabolism of the cell could be greatly reduced in amount without any effect on growth being apparent. This paper describes an instance of this sort; traces of selenite and molybdate, as well as iron, were found to be essential for the formation of an active formic dehydrogenase by strains of coliform organisms grown in a purified synthetic medium, although the first two factors, i.e. selenite and molybdate, had no influence on growth.

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EXPERIMENTAL

Organisms. That most commonly used was 1433, a strain of *Escherichia coli* obtained from Dr M. R. Pollock. Essentially similar results were obtained using two other strains, N.C.T.C. no. 86, and *Esch. coli* 8 from the collection of Dr C. B. van Niel, and two strains of *Aerobacter aerogenes*, N.C.T.C. no. 5936, and *Aero. aerogenes* 1, supplied by Dr E. F. Gale.

Purification of chemicals. Since conflicting results were obtained in the early experiments, in which the 8-hydroxyquinoline extraction technique described by Waring & Werkman (1944) was used, a more rigorous method of purification was resorted to. The individual chemicals employed in the preparation of the media were treated as described below. The techniques were designed so as to eliminate traces of impurities by distillation where possible, and also to make use of the capacity of $Fe(OH)_3$ to adsorb traces of selenite among other ions.

Certain general precautions were taken in all manipulations. Only Pyrex glassware was used; each item carefully cleaned immediately before use by rinsing first with ethanolic KOH, followed by distilled water, conc. HCl, and finally with glass-distilled water. Care was taken throughout to keep beakers and the necks of flasks covered with hardened filter paper or with cleaned beakers to avoid contamination with dust. The water and solvents used during the purifications were freshly distilled in an all-Pyrex still.

The purified chemicals were stored in cleaned, wide-mouthed Pyrex bottles, and kept in a clean desiccator to

avoid contamination. Stock solutions of $MgSO_4$, $FeSO_4$, H_2SeO_3 and Na_2MoO_4 were kept in Pyrex bottles protected from dust, and were made up freshly every 3 or 4 weeks as an additional precaution.

Ammonium dihydrogen phosphate. This was prepared from redistilled ammonia and phosphorus oxychloride as suggested by Hutner, Provasoli, Schatz & Haskins (1950). The salt was recrystallized from water, filtered off and dried at 100°.

Potassium chloride. 20 g. KCl were dissolved with heating in 55 ml. water containing 0·5 ml. 50% (w/v) $FeCl_3$. Approximately 6N redistilled NH_4OH was added until a precipitate of $Fe(OH)_3$ had been formed and the solution was alkaline. The precipitate was removed, and the solution shaken, while still warm, with 8-hydroxyquinoline in $CHCl_3$ solution to remove any trace of unprecipitated iron. After further extractions with portions of clean $CHCl_3$ to remove residual 8-hydroxyquinoline, the solution was cooled to a low temperature; the crystals were collected by filtration and dried at 100°.

Other chemicals. $NaNO_3$ and sodium succinate were purified by similar means; the latter was dried *in vacuo*. Glycerol was redistilled under reduced pressure. $MgSO_4$, $FeSO_4$ and ammonium molybdate were recrystallized. SeO_2 was prepared from selenium metal as described by Baker & Maxson (1939), and purified by sublimation. It dissolved readily in water to form selenious acid, which was not neutralized.

No special purifications of the substances investigated for ability to replace selenite and molybdate were undertaken. Analytical grade samples of NH_4VO_3, $K_2Cr_2O_7$, Na_2WO_4 and $Na_2SeO_4.10H_2O$ were used, while K_2TeO_3, $(NH_4)_2TeO_4$ and $UO_2(NO_3)_2.6H_2O$ were standard laboratory reagents.

Growth medium. The precise composition of the growth medium had no influence on the need for the presence of selenite and molybdate for the production of an active formic dehydrogenase. Thus neither wide variation of the concentration of inorganic constituents nor the use of different sources of carbon and energy had any effect on the results. Glucose, glucose/DL-lactate, succinate/L-glutamate and succinate/glycerol were investigated as carbon and energy sources. The medium generally used contained only easily purified substances and the concentrations of these were the least required to yield about 1 mg. dry cells/ml. under the conditions employed. Its composition was as follows: $(NH_4)H_2PO_4$, 0·0773 g.; KCl, 0·015 g.; sodium succinate, 0·2 g.; glycerol, 0·1 ml.; $MgSO_4.7H_2O$, 5·07 mg. ($\equiv$500 μg. Mg^{2+}); $FeSO_4.7H_2O$, 5 μg. ($\equiv$1 μg. Fe^{2+}); made up to 100 ml. with fresh, glass-distilled water. When added, both molybdate and selenite were at 1×10^{-7}M concentration.

In spite of failing to provide many trace elements, this medium appeared to be complete. Neither the growth nor the activity of cells was affected by the further addition of copper, cobalt, manganese and zinc. On the other hand, omission of iron resulted in a 100-fold reduction in cell yield, and omission of magnesium in at least a 1000-fold reduction (no visible growth was obtained).

Experiments were also carried out in which a nitrate-containing medium was used; it contained the above-mentioned minerals, but a higher $(NH_4)H_2PO_4$ content (0·4%, w/v) to raise its buffering capacity, 0·5% (w/v) $NaNO_3$, and 0·05% (w/v) glucose as carbon and energy source.

Growth conditions. The nitrate-free medium was made up immediately before use, and dispensed in 50 ml. lots in 500 ml. Erlenmeyer flasks. Appropriate quantities of selenite and molybdate solutions were added to each flask and the necks of the flasks were covered with suitably sized beakers. A layer of cotton wool was tied so as to cover the gap between the rim of the beaker and the neck of the flask. The inoculum was prepared by suspending a loopful of the growth from an 8 hr. 5% (v/v) yeast-autolysate agar slope in sterile, glass-distilled water, and one drop of this was added to each flask after autoclaving. The flasks were incubated on a horizontal shaker at 28–30°.

The nitrate-containing medium was made up in a similar way, but dispensed in 100 ml. lots in 200 ml. Erlenmeyer flasks. The cultures were also incubated at 28–30°, but were not shaken.

Preparation of washed suspensions. The cells were harvested at the point at which the cultures attained their maximum density, which was usually 15–20 hr. after inoculation. This was not strictly necessary for cultures grown in the nitrate-free medium, in which, as shown by Wooldridge, Glass & Knox (1936), the formic dehydrogenase activity remains roughly constant for a considerable period after growth has ceased. With the nitrate-containing medium, however, a peak in activity coincident with the cessation of growth was observed; thereafter the decay of formic dehydrogenase activity was rapid.

The cells were washed once in distilled water. This was found to be sufficient to avoid high blank O_2 uptakes and it was not found necessary to use glass-distilled water, or to take special precautions to prevent contamination with dust during washing, in order to demonstrate the effect of selenite or molybdate deficiency in the growth medium on the formic dehydrogenase activity of the cells. Waring & Werkman (1944) made a similar observation in their experiments on iron deficiency. The suspensions were adjusted to a concentration of 5 mg. dry wt./ml., using a Hilger or a Klett-Summerson Photoelectric Absorptiometer previously calibrated against suspensions of known dry wt.

Estimation of formic dehydrogenase activity. The rate of O_2 consumption in the presence of formate was taken as an index of the formic dehydrogenase activity of the cells (cf. Gale, 1939); no special precautions as to the purity of the reagents or cleanliness of the glassware were taken. Warburg manometers were used, the cups containing: cell suspension, 1 ml.; 0·1M-KH_2PO_4/Na_2HPO_4 buffer, pH 6, 0·5 ml.; 0·5M sodium formate (side bulb) 0·1 ml.; 20% (w/v) KOH (centre well) 0·2 ml.; water, 0·2 ml. The gas phase was air, and the temp. 30°. The rate of O_2 consumption in the absence of added substrate was low, and remained roughly linear for a period of 1 hr. In practice, therefore, the blank O_2 uptake was measured for 10 or 15 min. after equilibration, and the formate then tipped into the main compartment of the cup. O_2 consumption in the presence of substrate reached its highest rate after about 5 min., and was followed for 30–40 min.; it remained virtually linear for a period of at least 1 hr. at this temp. in contrast to the falling rate noted by Gale (1939) at 37°. O_2 uptake in the presence of glucose was measured similarly, except that the buffer used had a pH of 7.

On occasion, formic dehydrogenase was also measured by determining the rate of methylene-blue reduction in evacuated Thunberg tubes, whose contents were as follows: cell suspension, 0·5 ml.; 0·1M-KH_2PO_4/Na_2HPO_4 buffer,

pH 6, 1 ml.; water, 0·3 ml.; 0·5 M sodium formate (stopper), 0·1 ml.; 0·002 M methylene blue (stopper) 0·1 ml.

The results for blank O_2 uptakes, and for those in the presence of substrate (uncorrected for blank) are expressed as Q_{O_2} (μl. O_2 taken up/hr. by 1 mg. dry wt. of cells), or as Q_{MB} (μl. methylene blue reduced/hr. by 1 mg. dry wt. of cells, where 1 μmole = 22·4 μl.). Thus Q_{O_2} is equivalent to 0·5 Q_{MB}.

Cell-free preparations. These were made by tryptic digestion of the cells (Gale, 1939), and grinding with powdered glass.

RESULTS

Identification of factors needed to obtain active cell suspensions. This investigation arose from the observation that confirmation of the need for iron in formic dehydrogenase formation, demonstrated by Waring & Werkman (1944), could only be obtained if a mineral supplement containing copper, cobalt, manganese, zinc and molybdenum were added to the medium. In these experiments the nitrate-containing medium was used, purified by extraction with 8-hydroxyquinoline. Cells harvested from such media without the addition of the above-mentioned mineral supplement had a Q_{O_2} in the presence of formate of −20 or less, regardless of the amount of iron in the medium. If the supplement had previously been added, however, cells grown in the presence of sufficient iron had a Q_{O_2} with formate of about −100, and the effect of iron deficiency was dramatic. Hence it seemed clear that the supplement contained an ingredient which was required for the synthesis of formic dehydrogenase in the presence of adequate iron. This was identified as molybdate.

When the batches of chemicals used in these tests had been exhausted, even these results could no longer be duplicated except when the medium was made up with tap water. It was inferred, therefore, that some second factor, present in London tap water, and previously added to the medium as an impurity in the chemicals used, was involved in formic dehydrogenase formation. Attempts to isolate this from tap water were not successful. However, by treating boiler-scale with nitric acid, a solution in water was obtained that effectively replaced tap water as a means of inducing formic dehydrogenase synthesis, and by standard analytical procedures it was possible to show that the specific component belonged to the group of elements precipitated by hydrogen sulphide in acid solution, namely, bismuth, copper, cadmium, gold, platinum, mercury, arsenic, antimony, tin, molybdenum, selenium and tellurium. Solutions containing these elements were prepared, and selenite was found to be the only substance having any effect on formic dehydrogenase synthesis. It was clear by this stage that the procedure of extracting with 8-hydroxyquinoline was inadequate for purification of the medium, and the preparation of carefully purified reagents was undertaken. Results given below refer only to experiments in which media made up with such chemicals were used.

Experiments in purified media. Cells grown in a medium made with purified ingredients possessed a high formic dehydrogenase activity only if both selenite and molybdate were added, and sufficient iron had been supplied (Table 1). In the experiments described below, a medium containing 0·01 μg. Fe^{2+}/ml. was therefore used. In the great majority of tests neither selenite nor molybdate alone caused any increase in the formic dehydrogenase activity of the cells; therefore in the few instances where such an increase in activity was observed in the presence of only one of the two substances the effect was attributed to contamination of the medium or the glassware with a trace of the

Table 1. *Effect of inorganic factors in growth medium on formic dehydrogenase activity of* Esch. coli 1433

The manometer cups contained: cell suspension, 1 ml.; 0·1 M sodium plus potassium phosphate buffer, pH 6 for formate, pH 7 for glucose, 0·5 ml.; 0·5 M sodium formate or glucose (side bulb) 0·1 ml.; 20% (w/v) KOH (centre well) 0·2 ml.; water 0·2 ml.; gas phase, air; temp. 30°.

The Thunberg tubes contained: cell suspension, 0·5 ml.; 0·1 M sodium plus potassium phosphate buffer, pH 6, 1 ml; water, 0·3 ml.; 0·5 M sodium formate (stopper) 0·1 ml.; 0·002 M methylene blue (stopper) 0·1 ml. For further details see text.

Growth medium	Factors added to growth medium	Final growth (mg./ml.)	Blank $-Q_{O_2}$	$-Q_{O_2}$ glucose	$-Q_{O_2}$ formate	$\frac{Q_{MB}}{2}$ formate
Nitrate-free	Mg^{2+}	0·011	5	—	31	—
	Mg^{2+}, SeO_3^{2-}, MoO_4^{2-}	0·012	6	—	20	—
	Mg^{2+}, Fe^{2+}	0·92	10	108	12	—
	Mg^{2+}, Fe^{2+}, SeO_3^{2-}	0·91	8	—	18	—
	Mg^{2+}, Fe^{2+}, MoO_4^{2-}	0·96	5	—	15	—
	Mg^{2+}, Fe^{2+}, SeO_3^{2-}, MoO_4^{2-}	0·93	3	114	116	—
Nitrate-containing (0·06 M)	Mg^{2+}	0·051	5	—	8	—
	Mg^{2+}, SeO_3^{2-}, MoO_4^{2-}	0·046	4	—	10	—
	Mg^{2+}, Fe^{2+}	0·14	5	96	8	—
	Mg^{2+}, Fe^{2+}, SeO_3^{2-}	0·16	3	114	30	25
	Mg^{2+}, Fe^{2+}, MoO_4^{2-}	0·15	7	112	16	19
	Mg^{2+}, Fe^{2+}, SeO_3^{2-}, MoO_4^{2-}	0·15	2	102	108	112

other factor. That this should occur occasionally, in spite of the precautions taken, is not surprising in view of the very low concentrations in which these factors are needed. Thus maximal stimulation in the nitrate free medium was obtained when the concentration of selenite was between 3×10^{-8} and 1×10^{-7} M, and that of molybdate about 1×10^{-8} M (Fig. 1). In the nitrate-containing medium, in which the yield of cells was 0·1–0·2 mg./ml., as compared with nearly 1 mg./ml., stimulation by molybdate in the presence of excess of selenite was detectable at a concentration as low as 1×10^{-10} M, and was maximal at 3×10^{-9} to 1×10^{-8} M.

The effect of selenite and molybdate was seen only if the cells were grown in the presence of these ions. Experiments were carried out in which cells grown in the presence of only one of the two factors were incubated in manometer cups for 45 min. at 30° with the other factor at a concentration of 1×10^{-3} M. This treatment was without effect; whereas the Q_{O_2} in the presence of formate was −18 and −20 for cells freshly harvested from media deficient in selenite or molybdate respectively, the corresponding values for the cells after incubation with the substance previously deficient were −17 and −15.

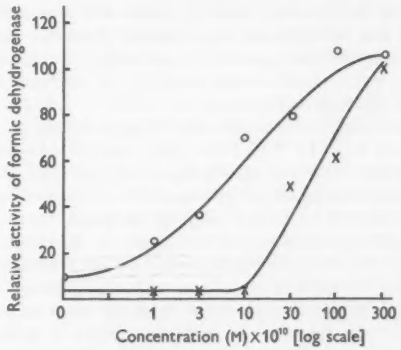

Fig. 1. Formic dehydrogenase activity of cells grown in media containing excess of either selenite or molybdate and varying amounts of the other substance. O—O, varying molybdate, selenite concentration = 1×10^{-7} M; ×—×, varying selenite, molybdate concentration = 1×10^{-7} M.

The rate at which cells took up oxygen if glucose were the substrate was approximately the same in all cases, and was independent of the formic dehydrogenase activity (Table 1).

Attempts to replace selenite and molybdate with other ions. As far as could be determined, the need for selenite and molybdate is highly specific, and no substitute was found.

Neither tellurate nor tellurite was able to replace selenite in the presence of excess of molybdate, even when their concentrations were 100 times that required for selenite to give full stimulation. Selenate had some activity at this concentration, but as the activity of several samples of selenate was raised to that of selenite by reduction on boiling in conc. hydrochloric acid, it is believed that the media to which selenate had been added contained some selenite as well, perhaps generated by a partial reduction of the selenate during autoclaving (Table 2).

Vanadate, chromate, tungstate and uranyl ions were tested for their capacity to replace molybdate in the presence of excess of selenite, but without success.

Effect of tungstate. Tungstate was found to have an action antagonistic to molybdate in the nitrate-containing medium, although no such effect was observed in the absence of nitrate. A strictly competitive relationship was observed between molybdate and tungstate if nitrate were present in the medium; the effect of molybdate was unaffected by 3 times, but annulled by 10 times its concentration of tungstate (Table 3). This relationship held good at least over the range 1×10^{-8} to 1×10^{-5} M. All the strains of *Esch. coli* that were used produce nitrite from nitrate during growth, and the antagonistic effect of tungstate was observed if the cells were grown in nitrate-free media to which 0·005 M sodium nitrite had been added after autoclaving; in

Table 2. *Inability of other ions to replace selenite and molybdate*

Formic dehydrogenase activity was measured manometrically.

Excess of MoO_4^{2-} (10^{-7} M) present				Excess of SeO_3^{2-} (10^{-7} M) present			
Ion added	Concn. (M)	$-Q_{O_2}$	Relative activity	Ion added	Concn. (M)	$-Q_{O_2}$	Relative activity
SeO_3^{2-}	10^{-7}	107	100	MoO_4^{2-}	10^{-7}	113	100
—	—	15	14	—	—	16	14
TeO_3^{2-}	10^{-5}	8	7	VO_3^-	10^{-5}	15	13
TeO_4^{2-}	10^{-5}	6	6	$Cr_2O_7^{2-}$	10^{-5}	16	14
SeO_4^{2-}	10^{-7}	16	15	WO_4^{2-}	10^{-5}	14	12
SeO_4^{2-}	10^{-5}	60	56	UO_2^{2+}	10^{-5}	24	21
SeO_4^{2-} (after reduction with HCl	10^{-7}	113	105				

Table 3. *Effect of tungstate and molybdate on formic dehydrogenase activity of* Esch. coli 1433

Formic dehydrogenase activity was measured manometrically and the results expressed as in Table 2.

Medium	WO_4^{2-} concn. (10^7 M)	MoO_4^{2-} concn. (10^7 M)	$-Q_{O_2}$	$\frac{WO_4^{2-}\text{ concn.}}{MoO_4^{2-}\text{ concn.}}$	Relative activity
Nitrate-free	0	1	135	—	100
	10	1	147	10	109
	100	1	127	100	94
Nitrate-containing (0·06 M)	0	0·1	98	—	100
	1	0·1	11	10	11
	1	0·3	77	3·3	78
	1	1	81	1	83
	10	0·1	6	100	6
	10	1	19	10	19
	10	3	94	3	96
	10	10	88	1	91
	100	0·1	11	1000	11
	100	100	125	1	128
Nitrite-containing (0·005 M)	0	1	157	—	100
	10	1	104	10	66
	100	1	28	100	16

this case a relatively higher concentration of tungstate was needed to neutralize the molybdate (Table 3). Tungstate (1×10^{-3} M) exerted no toxic effect on formic dehydrogenase if present in manometer cups during activity measurements.

Attempts to purify formic dehydrogenase. The isolation of the enzyme was attempted in order to elucidate the function of selenite and molybdate. It was found, however, in confirmation of Gale's (1939) results, that the enzyme was tenaciously bound to the cell fragments when the cells were broken by tryptic digestion or grinding with powdered glass. The only method found which appeared to bring the enzyme into solution was the butanol treatment described by Morton (1950); this, however, brought about such rapid inactivation that further purification of the enzyme was not achieved.

DISCUSSION

The effects of the deficiency of a far wider range of inorganic factors can be investigated if a medium prepared from specially purified chemicals is used, instead of relying on chelating reagents to remove unwanted ions from the solution. This method has not been widely used in the past, though Young, Begg & Pentz (1944), for example, used purified chemicals to investigate the inorganic growth requirements of *Esch. coli*. The technique used in the present investigation offered little difficulty, and once stocks of purified chemicals had been built up, the preparation of the medium took little more time than did that of the unpurified equivalent. Provided that proper care was taken to clean all glassware and to protect the medium at all stages from contamination with dust, consistent results were obtained if growth in the absence of iron were taken as index. Contamination with molybdate or selenite, reflected in relatively high Q_{O_2} values for cells grown with the addition of either factor alone, provides a more sensitive test of the reliability of the technique; in practice, such results were encountered only on three occasions.

The weight of a single cell of *Esch. coli* is approximately $2{\cdot}5 \times 10^{-13}$ g. From this it can be calculated that the effect of molybdate on formic dehydrogenase activity in the presence of adequate selenite can be detected when only 50 molecules of it are available for each cell in the culture. Full activity is observed when each cell could contain 5000 molecules, though it is not, of course, certain that all the molybdate available in the medium finds its way into the cells. Rather larger numbers of selenite molecules are required, but full activity is developed when 5000 to 10000 molecules are present per cell. It seems most unlikely, therefore, that the effects observed could be due to impurities in the preparations of these two factors, and it is of interest that Burk (1934) calculated that the number of molybdenum atoms required per cell for maximum activity of the nitrogen-fixing mechanism of *Azotobacter vinelandii* was about 10000. While the need for molybdenum in nitrogen fixation has been recognized since the work of Bortels (1930), this is the first time that a biological function has been ascribed to selenium, although the presence of this element in the ash of plants has been known for many years. Moreover, the need for molybdenum in formic dehydrogenase formation differs from that in nitrogen fixation in that vanadium, shown by both Bortels (1930) and Burk (1934) to be capable of replacing molybdenum in the latter reaction, has no activity in stimulating the production of formic dehydrogenase.

The measurement of rates of oxygen uptake in the presence of some oxidizable substance is admittedly an indirect way of estimating the quantity of the responsible dehydrogenase in cells. In the present instance, however, it seems highly probable that the Q_{O_2} values obtained do reflect the enzyme content of the cells in view of the following considerations. First, the Q_{O_2} in the presence of glucose was always high, no matter what was the corresponding value for formate. This indicates that the mechanism for oxygen activation in the deficient cells was unimpaired. Secondly, Q values calculated from measurements of the rate of methylene-blue reduction in the presence of formate agreed well with those obtained from the manometric experiment. Finally, it was found that the activity of cell-free preparations corresponded to that of the parent cell suspension. As noted by Gale (1939), there was an apparent increase in formic dehydrogenase of up to double that of the intact cells on disruption of the cell membrane either by tryptic digestion or grinding with powdered glass when normal cells formed the starting material. With deficient cells, having a Q_{O_2} (formate) of about 10, very little or no increase of activity was observed on breaking the cells. These results argue in favour of the contention that the Q_{O_2} values represent an acceptable measure of the formic dehydrogenase content of the cells.

Though the work described above seems sufficient to establish that molybdate and selenite have an essential function in the formation of formic dehydrogenase, it is not at present clear whether these substances act as such, or undergo some combination before their use. Nor is it known whether they are eventually incorporated in the enzyme molecule, or are involved more remotely in its synthesis. Observation of the fact that selenite and molybdate influenced the formic dehydrogenase activity of cells only if these substances had been present in the growth medium, and were without effect in short-term experiments (in which cells deficient in only one of the substances were incubated with selenite or molybdate) does not provide a distinction between these possibilities, although it can be taken to indicate that neither of these substances forms a loosely bound part of an enzyme complex. This is in accord with Gale's (1939) demonstration that the activity of his cell-free preparation of formic dehydrogenase from *Esch. coli* was not affected by dialysis. The problem could be resolved if the enzyme could be purified, but in spite of intensive efforts no success in this direction has been achieved.

Billen & Lichstein (1951) have reported that the formic dehydrogenase activity of a strain of *Esch. coli* grown in a simple synthetic medium was low unless a mixture of amino acids were also present during growth. Stimulation of activity in washed suspensions incubated with the amino-acid mixture was also observed, although the unspecific nature of the procedure followed for estimation makes these experiments hard to interpret. It would appear to be possible, however, that their basic medium contained inadequate selenite or molybdate, and that the effect of the amino acids might be due, in part at least, to contamination with these inorganic factors. In experiments in which many samples of A.R. grade chemicals were used for preparing the medium, the results obtained could be explained in terms of various degrees of contamination with these two substances.

Billen (1951) has also reported an inhibitory effect of nitrate on formic dehydrogenase formation, and here the antagonism between molybdate and tungstate in nitrate-containing media might be operative. Why this antagonism should be apparent only if nitrate or some reduction product of nitrate is present in the medium is not immediately obvious. It might be suggested that in spite of their general similarity, free tungstate and free molybdate are too different to be interchangeable in a biological system; if nitrate is also present in the medium, however, a biologically active complex might be formed between molybdate and some derivative of the nitrate. This, being larger than the free ion, might be less readily distinguished from the corresponding tungstate complex, so that a typical case of competitive inhibition might become demonstrable.

SUMMARY

1. The preparation of a purified culture medium for *Escherichia coli* is described.

2. In such media, selenite and molybdate, as well as iron, are required for production of formic dehydrogenase.

3. These factors are effective only if present in the growth medium, and have no effect on washed suspensions.

4. Suspensions of cells oxidize glucose rapidly, whether they contain an active formic dehydrogenase or not.

5. Selenite is not replaceable by selenate, tellurite or tellurate.

6. Molybdate is not replaceable by vanadate, chromate, tungstate or uranyl ions.

7. If the medium contains nitrate, tungstate competitively antagonizes the effect of molybdate. Tungstate is without effect in nitrate-free media.

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Countercurrent Distribution Studies on Anterior Pituitary Growth Hormone

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Since the isolation of a growth hormone preparation which behaved as a homogeneous protein by electrophoretic, diffusion and solubility criteria (Li, Evans & Simpson, 1945), a number of biological activities have been attributed to this protein (for a review of these activities see Young, 1953). The question of whether or not the growth-promoting as well as the other activities are indeed properties of the protein itself, or whether they might reside in smaller molecules, becomes increasingly pertinent in view of the recent extensive work on the adrenocorticotrophic hormone and on the active principles of the posterior lobe of the pituitary gland.

Thus 'protein' adrenocorticotrophic hormone fulfils the same physical criteria of purity as growth hormone, but its biological activity can be separated from the bulk of the protein material by a variety of techniques (see Cortis-Jones, Crooke, Henly, Morris & Morris, 1950; Astwood, Raben, Payne & Grady, 1951; Dixon, Moore, Stack-Dunne & Young, 1951; Hess, Harris, Carpenter & Li, 1951). The same situation is found in the case of the posterior-pituitary principles. van Dyke, Chow, Greep & Rothen (1942) isolated a protein which contained pressor, antidiuretic and oxytocic activities in the same ratios as those of standard posterior-pituitary powder. This protein was homogeneous by ultracentrifugal and solubility criteria but showed some inhomogeneity by electrophoresis. Many workers, however, have been able to separate an oxytocic principle from an antidiuretic-pressor principle (see Kamm, Aldrich, Grote, Rowe & Bugbee, 1928; Irving & du Vigneaud, 1938; Potts & Gallagher, 1944), and both principles have recently been shown to be peptides of simple composition (Livermore & du Vigneaud, 1949; Pierce & du Vigneaud, 1950; Turner, Pierce & du Vigneaud, 1951).

In the present investigation, preparations of growth hormone have been subjected to countercurrent distribution in order to determine whether they would behave as a single substance under these conditions and to discover whether or not the growth-promoting activity could be separated from the bulk of the protein by this means.

EXPERIMENTAL

Growth-hormone preparations. Most of the growth hormone used in this investigation was prepared in this laboratory from ox pituitaries by a modification (Reid, 1952) of the method of Wilhelmi, Fishman & Russell (1948). The material thus prepared does not always appear homogeneous during electrophoresis at pH 9·5. In addition, 25 mg. of a preparation were kindly supplied by Prof. C. H. Li, Berkeley, California, U.S.A., to Prof. F. G. Young. It was stated that the material behaved as homogeneous when studied by zone electrophoresis, on a chromatographic column and by end-group analysis.

Counter-current distribution. The apparatus was a 54-tube, all-glass apparatus (Quickfit and Quartz Ltd.), similar to that described by Craig & Post (1949). The volume of each phase was 35 ml. The solvent system was 2-butanol : aqueous *p*-toluenesulphonic acid, the acid concentration being approximately 0·005 M or 0·0025 M. The system was prepared by equilibrating equal volumes of the acid and 2-butanol. The 2-butanol was redistilled and was low in peroxides when tested as described by Newton & Abraham (1950). Theoretical curves were constructed as described by Craig & Craig (1950). For distribution, the material was first suspended in 1–2 ml. of water and then treated with 33 ml. of the aqueous *p*-toluenesulphonic acid saturated with 2-butanol (lower phase). All preparations studied went completely into solution under these conditions. After the

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first equilibration with 2-butanol (upper phase), a stable emulsion formed. This broke on centrifuging but left a thin layer of material at the interface, which was removed with a capillary pipette. In some experiments it was found necessary during the first ten transfers to break the emulsions in the first two or three tubes by centrifuging. For subsequent transfers about 20–30 min. usually sufficed for complete breakage of emulsions. Unless otherwise indicated, distributions were performed at 14–18°.

Estimation of contents of each tube after distribution. Although weight determination is the most general and the most reliable method of obtaining a complete countercurrent distribution curve, such a method is impractical where it is necessary to use extremely small amounts of material. In the case of distribution of small amounts of proteins it would seem that a most sensitive and inclusive method of analysis is a photometric ninhydrin procedure applied after hydrolysis of the protein fraction. The following method, similar to that of Newton & Abraham (1950), proved satisfactory. The phases in a tube were made miscible by addition of 4 ml. of ethanol, and 2 ml. samples were removed, placed in thin weighing shells (Craig, Hausmann, Ahrens & Harfenist, 1951) and evaporated to dryness. The sample in each shell was dissolved in 0·5 ml. of 6 N-HCl, which was then sucked into a small piece of soft-glass tubing (int. diam. 5–6 mm.), drawn out to a long fine tip at both ends. The tubes were sealed off and heated at 110–120° for 16 hr. The tips of the constrictions were then broken off and the contents blown into a shell and evaporated to dryness. Each sample was dissolved in 0·5 ml. of water, transferred by capillary pipette to a test tube, and subjected to the photometric ninhydrin procedure of Moore & Stein (1948). After development of the colour, samples were diluted with 10, 20 or 30 ml. of diluent before being read in a colorimeter (Evans Electroselenium Ltd., Harlow, Essex) against suitable blanks. When 50 mg. of protein were distributed through fifty-three transfers, the samples analysed contained 0–150 μg. of protein. The method was not absolutely quantitative because, to save labour, no rinsing was done at any stage. Since, with care, reproducible results can be obtained, the errors must be essentially constant.

In the determination of partition coefficients an indication of the amount of material in each phase was obtained by measuring the ninhydrin colour given by unhydrolysed material. With a system containing NH_4OH, the absorption at 275 mμ. was measured.

Recovery of material for biological assay. To recover material for determination of growth-promoting activity, the contents of desired tubes were combined, brought to pH 5–6 by addition of 0·1 N-NaOH and concentrated to 30–40 ml. at 0–10° in a rotary evaporator (Craig, Gregory & Hausman, 1950). If, during the concentration, the solution became cloudy, more NaOH was added until the solution was as clear as possible. The final pH of the concentrate was about 9. The solutions were then dialysed for 48 hr. against distilled water and freeze-dried.

Determination of growth-promoting activity. The increase in weight of hypophysectomized rats was used as a measure of growth-promoting activity (Li *et al.* 1945; Wilhelmi *et al.* 1948). Female rats (Wistar albino strain) were employed in most cases and were maintained on M.R.C. diet 41 (Short & Parkes, 1949). Tests were begun 15–21 days after hypophysectomy, and the rats were often used for a second test several weeks later. Growth hormone (dissolved in 0·9% NaCl and brought to about pH 9) was injected subcutaneously for 9 days, and the average weight of the rats on the 9th and 10th days was compared with their average weight during the 3 days before injection.

RESULTS

Choice of solvent system

The partition coefficients of a growth-hormone preparation *A* in several pairs of solvents in single tubes were investigated. With 2-butanol and either 0·1% (v/v) or 1% (v/v) acetic acid, 0·001 N-HCl or 0·1% (v/v) NH_4OH, the material remained almost completely in the aqueous phase. With 0·005 M and 0·05 M *p*-toluenesulphonic acid as the aqueous phase, however, approximately 50–60% and 70%, respectively, of this preparation went into the butanol phase.

Partition of growth-hormone preparations between 2-butanol and approximately 0·005 M p-*toluenesulphonic acid*

Fig. 1 shows the results of a 53-transfer distribution of 50 mg. of growth-hormone preparation *B*. A small amount of material at the interphase was removed from the first tube. It can be seen from Fig. 1 that there are at least two distinct components. At the completion of the experiment the tubes in which emulsions formed were numbers 1–10 and 46–50, the emulsions in tubes 1–10 being the more stable. With respect to the major component, the curve calculated for a theoretical distribution agrees well with that obtained experimentally. Preparation *A* gave a curve similar in all respects, except that a greater percentage of material was found in tubes 1–10.

After the above distributions had been completed, 22 mg. of preparation *C*, provided by Prof. C. H. Li, were distributed through twenty-four transfers. In this case a negligible amount of material at the interphase was present in the first tube. Less trouble with emulsions was encountered, although emulsions formed in two groups of tubes as before, but the emulsion in the lower-numbered tubes was the less stable. The analysis of the distribution is given in Fig. 2*a* and shows that the percentage of the component found in the low-numbered tubes is only about 3% of the total material represented by ninhydrin colour.

The yield of material recovered from tubes 18–24 was 9·4 mg., approximately 44% of theory. In the distribution of preparation *A*, 15 mg. (approximately 55% of theory) were recovered in tubes 43–52. To make certain that this somewhat low recovery was not due to a loss of material during dialysis, a concentrated dialysate from another distribution was analysed by the ninhydrin method.

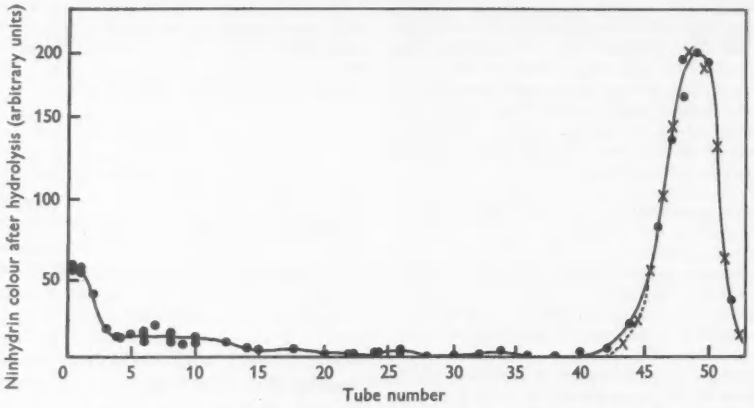

Fig. 1. Distribution curve of 50 mg. of growth hormone (preparation *B*) after fifty-three transfers. Solvent system—2-butanol:approx. 0·005 M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density equal to optical density multiplied by 100 with correction made for dilution of samples. ×, Theoretical values. Partition coefficient of major component, approx. 10; minor component, <0·04.

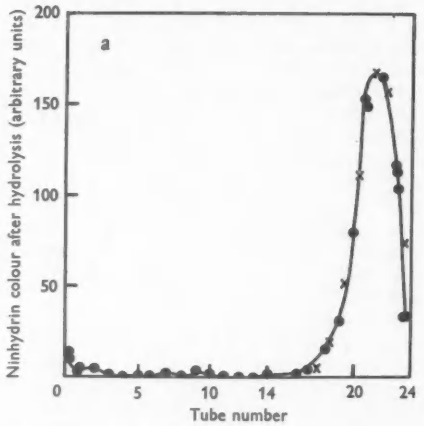

Fig. 2*a*. Distribution curve of 22 mg. of growth hormone (preparation *C*) after twenty-four transfers. Solvent system—2-butanol:approx. 0·005 M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density multiplied by 100 with correction made for dilution of samples. ×, Theoretical values. Partition coefficient of major component, approx. 10; minor component, <0·04.

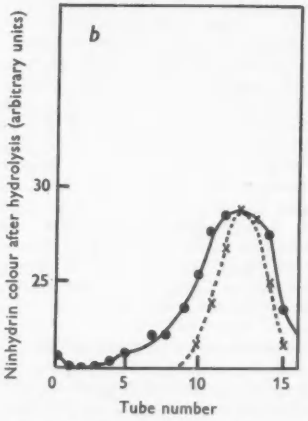

Fig. 2*b*. Distribution curve of 14 mg. of growth hormone recovered from main component of the distribution of preparation *B* (Fig. 1) after sixteen transfers. Solvent system—2-butanol:0·0022 M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density multiplied by 100 with correction made for dilution of samples. ×, Theoretical values for a partition coefficient of 4·8.

From 180 mg. of starting material less than 1 mg. was found in the dialysate of the main component. The low yield was probably due to the material passing over into the trap during freeze-drying.

Partition between 2-butanol and 0·0022 M p-toluenesulphonic acid

Although in the system with 0·005 M *p*-toluenesulphonic acid the main component distributes in good agreement with theory for a single substance, a better estimation of homogeneity could be made if conditions could be so chosen that the partition coefficient was close to unity. A preliminary experiment indicated that with an acid concentration of about 0·0025 M, a partition coefficient of about unity was obtained. A 14-mg. sample of the material recovered from the main peak of the distribution of preparation *B* in the first solvent system was re-

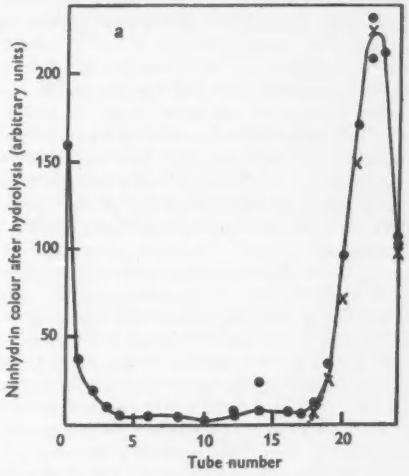

Fig. 3*a*. Distribution curve of 40 mg. of growth hormone (preparation *E*) after twenty-four transfers. Solvent system—2-butanol: 0·0044 M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density multiplied by 100 with correction made for dilution of samples. ×, Theoretical values. Partition coefficient of major component, approx. 10; minor component, <0·04.

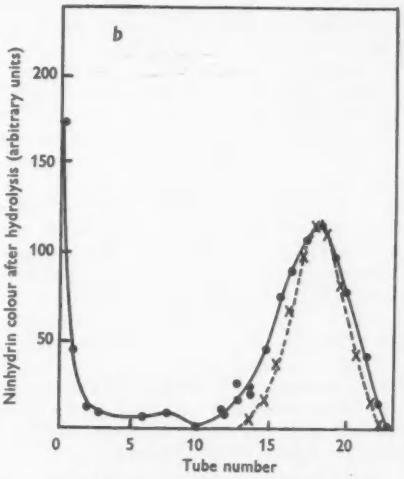

Fig. 3*b*. Distribution curve of 40 mg. of growth hormone (preparation *E*) after twenty-four transfers. Solvent system—2-butanol: 0·0022 M *p*-toluenesulphonic acid. ●, Arbitrary units of colour density multiplied by 100 with correction made for dilution of samples. ×, Theoretical values for a partition coefficient of 3·8; partition coefficient of minor component, <0·04.

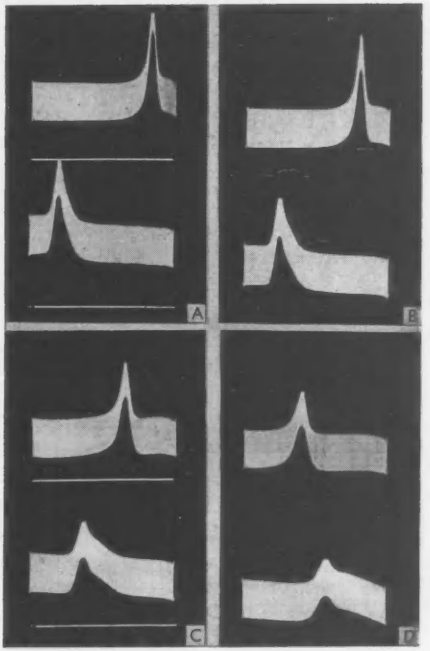

Fig. 4. Electrophoretic diagram of preparation *E*. pH 9·5, glycine buffer, ionic strength 0·1. *A*, *B*, *C* and *D*, 150, 185, 300, 480 min., respectively. Top: ascending boundary; bottom: descending boundary.

distributed in a system with 0·0022 M acid (the exact molarity was determined by titration of the aqueous phase before its equilibration with 2-butanol). The distribution curve is given in Fig. 2*b* and showed the partition coefficient to be greater than had been expected and that the curve had spread on both sides of the theoretical curve.

To examine the possibility that the deviation from the theoretical might have been due to degradation during the recovery of the material from the first distribution, 40 mg. of another growth-hormone preparation (*E*) were distributed through twenty-four transfers in the same system (0·0022 M acid). As a control, a second sample was distributed at the same time in 0·0044 M acid in the other half of the apparatus. The curves are given in Figs. 3*a* and 3*b*, and show that deviation from the theoretical again occurred at the lower acid concentration.

It is possible that the ninhydrin colour in the first few tubes might have been due to traces of ammonia passing into the counter current apparatus with the upper phase. The ammonia might then have been extracted by the lower phase. Determination

of the ninhydrin colour of samples from these tubes before and after hydrolysis showed the expected increase in colour following hydrolysis. Also the formation of emulsions in these tubes would be unlikely if the nitrogen present were only in the form of traces of ammonia.

A sample of preparation *E* was subjected to electrophoresis (Perkin-Elmer micro-electrophoresis apparatus, Perkin-Elmer Corp., Norwalk, Connecticut, U.S.A.) at pH 9·5, which is close to pH values used in the study of homogeneity of growth-hormone preparations (Li *et al.* 1945; Wilhelmi *et al.* 1948). This was carried out to see if the two components found in this preparation by counter-current distribution could be clearly separated by the electrophoresis. The pattern obtained is given in Fig. 4 and shows that no clear separation occurred.

Biological activity of fractions obtained by distribution

Preliminary experiments showed that a fair proportion of biological activity could be retained when growth hormone was distributed between 2-butanol and 0·005 M *p*-toluenesulphonic acid if dialysis were used as a means of removing the acid. The partition coefficient (k) of the biologically active material was then investigated. A 20 mg. sample of preparation *B* was equilibrated in a test tube between 20 ml. of each phase. In order to make sure that the activity was not concentrated in the material at the interphase, and was thus being discarded at the beginning of the distribution, this material was left with the lower phase, approximately 90 % of the upper phase being drawn off. If the activity were in the main component (k of approx. 10, as shown by the distribution curves), a total of about 20 % of the activity should remain with the lower phase and interphase fraction. These experiments were carried out at 0–4°. The assay results are in Table 1, section 1, and show that, at the dose employed, the upper-phase material gave twice the increase in weight as did the material from the lower phase. Comparison with a dose/response curve, determined with the less-pure preparation (*A*), indicated the activity of the lower-phase fraction to be about 20 % of the upper-phase fraction.

Table 1. *Growth-promoting activity of preparations after distribution*

Preparation	No. of animals	Daily dose (μg.)	Mean increase in wt. (g.)	Standard error of the mean	Fisher's *P* relative to saline control
Section 1*					
Preparation *B*, control treated with 0·005 M *p*-toluenesulphonic acid	3	100	19	±2·3	—
Preparation *B*, after equilibration between the two phases of the system, material from both phases	5	100	17	±1·3	—
Preparation *B*, treated as above, upper phase	4	100	20	±1·1	<0·001†
Preparation *B*, treated as above, lower phase plus 10 % upper phase	5	100	9	±0·9	—
Section 2					
Preparation *A* (material from tubes 43–52), 53-transfer distribution	6	100	14	±0·8	<0·001
Saline control for above	3	—	−2	±1·4	—
Preparation *C*, untreated	4	50	22	±3·4	<0·02
Preparation *C* (material from tubes 18–24), 24-transfer distribution	4	100	21	±0·7	<0·001
Saline control for above	2	—	2	±1·5	—
Preparation *D*, untreated	4	50	17	±0·8	<0·01
Preparation *D** (material from tubes 7–10), 9-transfer distribution	4	50	14	±0·9	<0·01
Saline control for above	2	—	0	±2·5	—
Section 3					
Preparation *E*, untreated	8	50	15	±1·4	<0·001
Preparation *E* (material from tubes 15–23), 24-transfer distribution, 0·0022 M acid	9	50	9	±1·4	<0·01
	6‡	100	15	±1·3	—
Preparation *E* (material from tubes 20–24), 24-transfer distribution, 0·0044 M acid	9	50	10	±1·5	<0·01
Saline control for above	6	—	2	±0·5	—

* Experiments carried out at 0–4°; all other experiments at room temperature, 17–18°.
† Fisher's *P* of upper phase value relative to lower phase value.
‡ Experiments carried out on rats used first as saline controls for this section.

It was then found that samples isolated from each of the main peaks in three experiments with different preparations possessed growth-promoting activity. The results are given in Table 1, section 2. In another experiment inactivation apparently occurred.

Results of bio-assay of growth hormone distributed between 2-butanol and 0·0025 M *p*-toluenesulphonic acid are given in Table 1, section 3, and demonstrate that the material recovered from the main peak retained a high proportion of activity.

DISCUSSION

The data show clearly that a large proportion of the growth-promoting activity of the preparations is found in the major component. The other component, however, has not been tested for biological action and it is possible that it may possess some activity. Although some loss of growth-promoting activity appears to take place during the distribution or recovery for assay, the material is relatively stable. In some experiments it was exposed to the solvent system for up to a week at 14–18°.

The molecular weight of growth hormone in the systems used for distribution is unknown. Recent measurements in phosphate buffer, pH 2·3, by Li & Pederson (1953) indicate a molecular weight of about 45 000, the same as in neutral solution. The fact that counter-current distribution can be successfully applied to growth hormone makes it appear that the technique will be of value in studies of the purity and perhaps in the isolation of other proteins of comparable molecular weight.

With insulin, Harfenist & Craig (1952) have demonstrated the existence of one major component with, in some preparations, varying amounts of closely related components. The solvent system employed was 2-butanol: 1% (0·077 M) dichloroacetic acid. With the system, 2:4:6-collidine: water, Hess, Carpenter & Li (1952) showed that 'protein' adrenocorticotropic hormone (mol.wt. 20 000) did not distribute as a single substance, and that biological activity was concentrated in a fraction of the material.

It is interesting to note the extremely low concentration of *p*-toluenesulphonic acid (0·005 M) in the system in which growth hormone has a partition coefficient of about 10. A similar system with *n*-butanol as the upper phase has been used for the purification of vasopressin (Turner *et al.* 1951), but the concentration of *p*-toluenesulphonic acid necessary to give a partition coefficient of unity was 0·1 M. With corticotropin *B* concentrates Kuehl, Meisinger, Brink & Folkers (1953) have obtained a favourable partition coefficient with 0·03 M trichloroacetic acid; the same range of concentration of other organic acids was used in the distribution studies of Payne, Raben & Astwood (1950) on adrenocorticotropic hormone. The reason for the relatively high solubility of growth hormone in the organic phase with such a low concentration of the acid is probably related to the presence of the large number of basic groups (approx. 58, Li & Evans, 1948) in growth hormone.

In the system with an acid concentration of 0·005 M the curve of the main component agrees well with theory for a single substance. However, the use of 0·0022 M acid in an attempt to get a more favourable partition coefficient for study of homogeneity led to non-theoretical behaviour, the curve showing a tendency to spread on both sides of the theoretical curve. Three possible reasons for non-theoretical behaviour are the presence of other components or transformation products, a change in partition coefficient with changing concentration of the solute, or the fact that equilibrium in the distribution of the solute between the phases might not be obtained before each transfer (Craig & Craig, 1950). It seems unlikely that the last two reasons would apply only at the lower concentration of acid. Further work in a different solvent system will be necessary to see if more than one component is represented by the main peak. It is possible that with an acid concentration of 0·0022 M not enough acid is present to form the most stable possible complex of *p*-toluenesulphonic acid with the many basic groups of growth hormone. A number of complexes with slightly different partition coefficients might then exist in equilibrium. At a concentration of 0·0022 M acid, it is of interest that the number of acid equivalents in one tube is about equal to the number of basic equivalents in 50 mg. of growth hormone.

SUMMARY

1. Countercurrent distribution of preparations of growth hormone in the system 0·005 M *p*-toluenesulphonic acid: 2-butanol produced at least one major and varying amounts of a minor component. The best preparation, supplied by Prof. Li and stated to be homogeneous by electrophoresis, chromatographic column and end-group analysis, contained only 3% of the minor component.

2. Growth-promoting activity was present in the major component, the distribution curve of which agreed well with the theory for a single substance.

3. With 0·005 M *p*-toluenesulphonic acid, the major component had a partition coefficient of about 10. A system with 0·0022 M *p*-toluenesulphonic acid was used in an attempt to obtain a more favourable partition coefficient for the study of homogeneity of the major component. In this case the distribution curve of the major component was wider than the theoretical curve. Possible

reasons for this deviation from the theoretical are discussed.

4. A comparison between the distribution curve of a preparation of growth hormone and its electrophoresis pattern is presented.

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Transfer Reactions Catalysed by some Sucrase Preparations

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In view of recent observations on the action of sucrase preparations from various sources (mainly yeasts and moulds, e.g. see Edelman, 1950; Bacon & Edelman, 1950; Blanchard & Albon, 1950; Fischer, Kohtès & Fellig, 1951; Bacon, 1952; Wallenfels & Bernt, 1952; White & Secor, 1952; Bealing & Bacon, 1953; Bealing, 1953) the existence of enzymes capable of catalysing solely the hydrolysis of sucrose to glucose and fructose is becoming increasingly doubtful. It appears that the fundamental action of sucrases may be to transfer fructose residues to suitable acceptors with consequent production of mono-, di- and oligo-saccharides. Some of the implications of fructose transfer have been discussed by Edelman & Bacon (1951) and Bealing & Bacon (1953).

This paper describes experiments with yeast- and mould-sucrase preparations, involving the use of radioactive sugars, which experiments further elucidate the fructose-transferring capacity of these enzymes. Some of the results described here are an extension and amplification of those communicated to the Biochemical Society on 24 October 1952 (Edelman & Bealing, 1953).

MATERIALS AND METHODS

Enzyme and yeast preparations

Dialysed mould sucrase. This was prepared from *Aspergillus oryzae* or *Penicillium spinulosum* using the Hughes' bacterial press by the method of Bealing & Bacon (1953). Alternatively, a 'Takadiastase' preparation, obtained from

Parke, Davis and Co. Ltd., London, was used. This was a powder prepared from *Aspergillus sp.* (probably *A. oryzae*) and standardized for use in analytical chemistry. Solutions were made in distilled water and dialysed for at least 12 hr. against distilled water before use.

Yeast sucrase. An invertase concentrate derived from yeast, and obtained from British Drug Houses Ltd., London, was used. It was dialysed against distilled water for at least 4 hr. before use.

Candida krusei. A culture of this sucrase-free yeast was obtained from the Brewing Industry Research Foundation, Nutfield, Surrey. Subcultures were maintained on malt agar. A liquid medium containing 1% Difco yeast extract and 3% (w/v) glucose in tap water was inoculated and incubated in flat bottles at 25° for 4 days. The liquid was centrifuged, the cells washed three times by suspension in water and centrifuging, and packed hard by high-speed centrifuging to give a moist, crumbly cake. The weight of packed cells was about equal to that of the glucose originally present in the medium. Thick suspensions of this preparation rapidly fermented glucose and fructose, with copious production of CO_2 after several minutes. Sucrose was not attacked even on prolonged incubation (up to 30 hr.).

Enzyme and yeast preparations were stored at 3°.

Radioactive sugars

Uniformly labelled [^{14}C]glucose was prepared in this laboratory by photosynthesis from $^{14}CO_2$, using tobacco leaves (Porter & Martin, 1953). Uniformly ^{14}C-labelled fructose prepared by a similar method was purchased from the Radiochemical Centre, Amersham, Bucks. The radioactivities of these sugars were about 5·5 and 50 μc/mg., respectively, estimated at infinite thickness after conversion into $BaCO_3$. They were diluted with inactive glucose (A.R.) or fructose (microbiological reagent) to give suitable radioactivities.

Paper-partition chromatography

This was carried out by the descending method using Whatman no. 1 filter paper and butanol:acetic acid:water (Partridge, 1948).

Samples of enzyme digests were inactivated by previously adding 5 μl. 0·01 M-$HgCl_2$ to the position on to which they were to be delivered (Bealing & Bacon, 1953).

Sugars were hydrolysed on the dried paper by spraying with an aqueous solution (8–10%, v/v) of yeast sucrase and drying at room temp. This was sufficient to hydrolyse entirely, to glucose and fructose, as much sucrose as would run satisfactorily as a discrete spot (i.e. about 400 μg. at room temp.).

Sugars were detected by the benzidine/trichloroacetic acid or phloroglucinol spraying reagents (Bacon & Edelman, 1951; and Horrocks & Manning, 1949, respectively).

Estimation of sugars from the paper

Sugar solutions were applied using an Agla Micrometer Syringe (Burroughs Wellcome and Co., London). After development, the paper was extracted with water at 75–80° for 15 min., shaken, and the resultant suspension filtered. Total fructose in a sample of the filtrate was estimated by a resorcinol/HCl method (see Bacon & Edelman, 1951), and reducing sugar by the method of Nelson (Somogyi, 1937; Nelson, 1944).

Estimation of radioactivity

Before and after development of the chromatogram, radioactivity in the sugar spots was estimated by applying a thin end-window Geiger-Müller counter, type EHM 2 (General Electric Co., London), directly to the paper; the counts/min. were obtained by using a scaler, type 1009 A (Dynatron Radio Ltd., Maidenhead). Daily variations in the counter were corrected by using a factor calculated from the counts/min. of a ^{14}C-labelled plastic block of activity

Table 1. *Estimation of radioactivity from developed chromatograms of* ^{14}C*-labelled sugars*

Activity estimated as described under Materials and Methods.

Sugar applied (μg.)	Before development		After development	
	Counts/min.	Counts/min./μg.	Counts/min.	Counts/min./μg.
A. Glucose (spots counted entire on one surface)				
8·25	3038	368	2780	337
16·5	6089	369	5460	331
B. Glucose (spots cut into four pieces. Sums of pieces counted on one surface)				
50	297	5·9	326	6·5
100	629	6·3	623	6·2
150	982	6·5	930	6·2
200	1297	6·5	1230	6·2
	Average	6·3	Average	6·3

C. Sucrose (spots cut into four pieces. Sums of pieces counted on both surfaces)

	Surface I (*a*)	Surface II (*b*)	From (*a*) and (*b*)	Surface I (*c*)	Surface II (*d*)	From (*c*) and (*d*)
15·2	301	340	21·1	328	318	21·3
30·2	611	634	20·5	592	618	19·9
45·6	910	937	20·3	1043	924	21·5
60·8	1166	1229	19·7	1250	1220	20·3
76·0	1505	1509	19·9	1672	1511	20·9
		Average	20·3		Average	20·8

1·0 μc/g. Unless otherwise stated, radioactivities are expressed as counts/min., corrected for lost counts due to counter paralysis time and for background, estimated directly from the paper. The weight of the paper was about 9 mg./cm.^{2}; no corrections were made for differences in self-absorption of the radiation caused by variations in paper thickness: the weights of twenty 4 cm.^{2} samples lay between 34·1 and 37·3 mg., average 35·4 $\pm$ 0·19 mg.

The diameter of the applied spot was never greater than about 1 cm. (equivalent to a single application of 5 μl.); its radioactivity was estimated by a single count (or the average of single counts on both paper surfaces).

At first only those developed spots of diameter >2 cm. were cut into pieces, each small enough to be covered by the counter end-window (diameter 2·3 cm.). It was found, however, that recovery after development was low (80–90% of the counts/min. applied) unless all the spots were cut into at least four pieces (see Table 1 *A*, *B*). This practice was subsequently adopted. It was also considered desirable to count both surfaces of each chromatogram, taking the average as the final value, as differences between surfaces sometimes exceeded 10% of the mean (Table 1, *C*); this was done only when the most accurate values obtainable were necessary, as the increased tedium of counting many spots each cut into several pieces was otherwise considered unjustified.

Counting periods varied from 1 min. (>1000 counts/min. source) to 5 min. (<100 counts/min. source). Recoveries after development nearly always lay between 90 and 110% of the counts/min. applied, and usually between 95 and 105%. Table 1 shows some results illustrating this method of estimation.

The considerable advantage of the method is its relative rapidity; it eliminates the necessity for elution from the paper and consequent difficulties in handling and counting very small quantities of material. Inaccuracies are normally insignificant and can be attributed to (*a*) the closeness of the end-window to the source; this also increases errors caused by variation in sensitivity over the surface of the window (Putman & Boxall, 1947), (*b*) occasional significant variations in activity between the two surfaces of a chromatogram and (*c*) slight variations in paper thickness.

Gradient-elution chromatography

The method is described by Bacon & Bell (1953) and by Alm, Williams & Tiselius (1952). It consists of eluting charcoal columns with solutions of progressively increasing ethanol concentration. The apparatus was arranged so that aqueous ethanol dropped into a reservoir, initially of distilled water, at the same rate as the solution in the latter passed through a charcoal-Celite column.

Activated Charcoal (British Drug Houses Ltd., London) was mixed with an equal weight of Celite 535 (Johns Manville Co., Ltd., London) (Whistler & Durso, 1950). The mixture was suspended in distilled water, filtered on a Büchner funnel, and dried at 105°. Columns were packed with the powder either dry or as a slurry in water. They were washed with distilled water until the effluent, at first acid, became neutral. Sugar solutions were then applied and fractions collected by a Shandon Automatic Fraction Collector (Shandon Scientific Co., London).

Gradient-elution chromatography was found to have greater resolving power for the sugar mixtures used than did the stepwise method described by Whistler & Durso (1950).

RESULTS

The action of mould sucrase

The results of the action of mould sucrase on sucrose are illustrated in Fig. 1. Some aspects of this reaction have been discussed in detail by Bealing & Bacon (1953). Table 2 shows the effect of adding glucose at the start of the reaction; both the formation of oligosaccharides and the disappearance of sucrose as estimated by combined fructose analysis were depressed (cf. Bealing, 1953). Addition of ^{14}C-labelled glucose to the reaction mixture (Table 3) caused radioactivity to appear in the sucrose spot. Boiled enzyme induced no detectable reaction in a mixture of sucrose and radioactive glucose.

Table 2 shows also the effect of adding free fructose; at the concentration investigated this did not greatly affect the formation of oligosaccharides, but appeared to retard sucrose disappearance. The use of labelled fructose showed that this sugar when added at the start of the reaction, also caused the sucrose spot to become radioactive (Table 4).

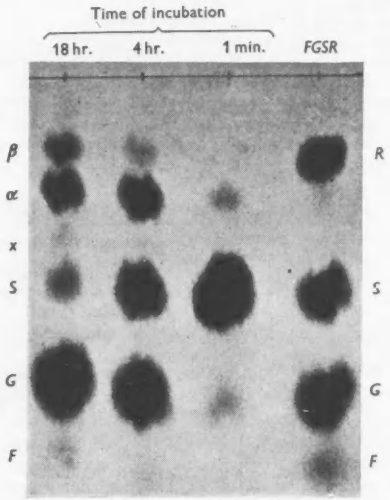

Fig. 1. The action of mould sucrase on sucrose. Chromatogram of 5 μl. samples of a reaction mixture at 20° containing 50 mg. sucrose, 0·9 ml. 0·05 M sodium acetate buffer, pH 5·0, and 0·1 ml. 10% (w/v) 'Takadiastase'. *FGSR*, reference sugar mixture containing 50 μg. fructose, 50 μg. glucose, 100 μg. sucrose and 150 μg. raffinose; *F*, fructose; *G*, glucose; *S*, sucrose; *R*, raffinose; *x*, probably a disaccharide containing glucose residues; α, a trisaccharide containing *G* and *F*, β, probably a tetrasaccharide containing *G* and *F*.

Table 2. *Some effects caused by the presence of added glucose or fructose on the action of mould sucrase on sucrose*

Each reaction mixture at 20° contained 500 mg. sucrose and 2·5 ml. 2% (w/v) 'Takadiastase' in a total volume of 5·0 ml. 0·05M sodium acetate buffer, pH 5·0. The appropriate mixtures also contained 250 mg. monosaccharide. Sugars were estimated from paper chromatograms. Results are expressed as mg. combined fructose/ml.

		Time of incubation (hr.)		
Substrates	Sugars estimated	0	5	20
Sucrose	Sucrose	53·2	31·4	10·2
	Oligosaccharides	—	15·7	28·3
Sucrose + glucose	Sucrose	54·2	37·8	17·3
	Oligosaccharides	—	9·9	21·0
Sucrose + fructose	Sucrose	53·2	36·6	19·9
	Oligosaccharides	—	13·4	26·7

Table 3. *The action of mould sucrase on sucrose and radioactive glucose*

A. Total activities. The reaction mixture contained 17·8 mg. sucrose, 9·8 mg. radioactive glucose with total activity about 5 μc, 0·1 ml. 0·05M sodium acetate buffer, pH 5·0, and 0·1 ml. enzyme preparation from *A. oryzae*. Incubation was carried out in an Agla syringe at 20°. Samples (3 μl.) were delivered on to filter-paper sheets. Results are expressed as counts/min. in the sugar spot.

	Time of incubation (hr.)						
	2 min.	0·5	1	2	4·25	18	28
Before development (total applied to paper)	5171	5468	5162	5196	5245	5400	5700
After development							
Glucose spot	4761	4921	5101	4448	4792	4950	5550
Sucrose spot	33	369	357	485	494	67	21
Spot x	7	5	4	30	48	52	38
Spot α	0	5	10	25	65	47	18
Spot β	—	—	—	—	6	6	8
Total	4801	5300	5472	4988	5405	5122	5635

B. Specific activities. Same reaction mixture as for *A*. Values deduced from paper chromatogram analyses combined with above table. Results are expressed as counts/min./μg. of free or combined glucose.

Glucose spot	39	36	34	30	26	21	20
Sucrose spot	0·3	2·3	3·5	6·5	10	5·6	3·2
Spot α	—	1·4	1·3	2·2	4·2	6·7	16

Table 4. *The action of mould sucrase on sucrose and radioactive fructose*

A. Total activities. The reaction mixture contained 18 mg. sucrose, and 11·6 mg. radioactive fructose with total activity about 6 μc. Experiment performed as that described in Table 3. Results are expressed as counts/min. in the sugar spot.

	Time of incubation (hr.)					
	5 min.	0·5	1	2	4·25	18
Before development (total applied to paper)	5384	5738	6083	5459	5953	6000
After development						
Fructose spot	5760	5430	5705	5470	5330	5070
Sucrose spot	26	80	144	252	357	576
Spot x	—	—	—	—	—	28
Spot α	9	3	13	11	40	74
Spot β	—	—	7	8	11	10
Total	5795	5513	5869	5741	5738	5758

B. Specific activities. Same reaction mixture as for *A*. Values deduced from paper chromatogram analyses combined with above table. Results are expressed as counts/min./μg. of free or combined fructose.

Fructose spot	36	34	33	31	29	24
Sucrose spot	0·2	0·7	1·4	3·0	6·0	14
Spot α	—	—	—	—	1·0	2·5

Table 5. *The identity of the radioactive sugars incorporated into oligosaccharides synthesized by mould sucrase*

The reaction mixture contained 15 mg. sucrose, 10 mg. radioactive glucose or fructose with total activity about 5 μc, 0·12 ml. 0·05 M sodium acetate buffer, pH 5·0, and 0·08 ml. enzyme preparation. Incubation was carried out in an Agla syringe at 20°. Samples (4 μl.) were delivered on to a filter-paper sheet after 2 hr. for the purpose of two-dimensional chromatography. Sugars were hydrolysed after the first development. No attempt at accurate quantitative estimation was made. Results are expressed as counts/min. in the sugar spot.

	Spot β	Spot α	Sucrose	Glucose	Fructose
Added radioactive glucose		Enzyme preparation from *P. spinulosum*			
1st development	83	500	450	4800	—
2nd development					
Derived glucose	202	582	484	4800	—
Derived fructose	—	4	15	—	11
Added radioactive fructose		Enzyme preparation from *A. oryzae*			
1st development	7	27	321	—	5250
2nd development					
Derived glucose	2	7	4	—	—
Derived fructose	3	12	298	—	4991

Table 6. *Investigation of the components giving rise to the 'sucrose' spot after incubation of mould sucrase with a mixture of sucrose and radioactive fructose*

For description of the experiment see text. Components *A* and *B* were separated by gradient elution.

	Total fructose content (mg.) (*a*)	Total glucose content (mg.) (*b*)	Ratio of glucose: fructose from (*a*) and (*b*) (*c*)	Counts/min. estimated from filter paper: Per μg. hexose (*d*)	Total, from (*a*) + (*b*) and (*d*) (*e*)	Fraction of total in mixture, from (*e*) (%)
Component *A*	5·7	0·03	0·00	15·9	91×10^3	92
Component *B*	7·8	7·8	1·0	0·5	$7{\cdot}8 \times 10^3$	8

Two-dimensional chromatography, with complete hydrolysis of the saccharides after the first development, showed that the incorporated activity was in the expected glucose or fructose parts of the molecules corresponding to the radioactive sugar added (Table 5).

The glucose-labelled material represented by the sucrose spot arising from the products of the enzymic action on a mixture of sucrose and labelled glucose was shown to be sucrose as follows. Syrup (137 mg.), consisting mainly of radioactive glucose, but contaminated with a little inactive fructose, was dissolved with sucrose (100 mg.) in 0·5 ml. 0·05 M sodium acetate buffer, pH 5·0; 0·5 ml. mould enzyme was added and the mixture was incubated at 22°. After 2·25 hr. the tube containing the digest was placed in a boiling-water bath and left for 5 min. The sugars were separated by the method of Whistler & Durso (1950) using a charcoal-Celite column (34 × 300 mm.) with aqueous ethanol as eluent. The fractions containing material giving rise to a spot in the sucrose position on a paper chromatogram were combined, evaporated to dryness under reduced pressure, and crystallized from aqueous ethanol. The crystals were washed with ethanol and dried. The first crop weighed 33·1 mg. X-ray powder photographs of this material and of a known sample of sucrose, crystallized in a similar manner, were indistinguishable. The activity of the labelled sucrose after crystallization and recrystallization was 33·2 and 33·3 counts/min./μg., respectively.

When labelled fructose was added to an enzyme–sucrose mixture, radioactivity became incorporated into the sucrose spot less rapidly than when labelled glucose was added under similar conditions. This activity, however, eventually reached a higher level (Tables 3 and 4). The activity was not due to incorporation of labelled fructose into sucrose, but to the formation of a sugar with similar R_F. This sugar, which was shown to consist only of fructose residues, was isolated and identified as follows.

A solution containing 50 mg. sucrose, 50 mg. radioactive fructose with total activity about 20 μc, 0·25 ml. 0·05 M sodium acetate buffer, pH 5·0, and 0·25 ml. 2 % (w/v) 'Takadiastase' was incubated at 20°. After 14 hr. 3 drops of 0·01 M-$HgCl_2$ were added and the sugars separated on a charcoal-Celite column (34 × 300 mm.) using gradient elution with 75 % (v/v) aqueous ethanol dropping into water (2 l.). Fractions were analysed both for sugar content and radioactivity. Appropriate fractions were combined and evaporated at reduced pressure to syrups of small volume.

Of the emergent sugars, two were investigated; component *A* which followed the monosaccharides, and component *B* which followed *A*. The syrups were made up to 2·6 ml. with water, and samples measured with an Agla syringe were chromatographed and analysed. Both sugars had the same R_F as sucrose on paper chromatograms. The results of the analyses are given in Table 6.

The action of yeast sucrase

The results of the action of yeast sucrase on sucrose are shown in Fig. 2. The effect of adding free glucose or fructose at the start of the reaction is shown in Table 7. Results of an experiment involving the use of labelled glucose are shown in Table 8; the activity values for sucrose in this table, although positive, showed no trend during the course of the reaction and were almost certainly due to slight trailing of the labelled glucose. There was significant incorporation of activity into spot I.

Similar experiments using labelled fructose demonstrated the incorporation of activity into the sucrose spot, but into no other oligosaccharides (Table 9).

Two-dimensional chromatography, with complete hydrolysis of the saccharides after the first development, showed that the activity was in the expected glucose or fructose parts of the molecules corresponding to the radioactive sugar added (Table 10).

That the formation of the material represented by spot I was entirely dependent on the presence of free hexose as well as sucrose was shown by eliminating hexose from the reaction mixture immediately it was liberated, by adding *C. krusei*, a yeast which lacks sucrase and thus will not ferment sucrose. Under these circumstances the action of yeast sucrase on sucrose did not give rise to free glucose or fructose (except in traces), or to spot I; spots II and III were formed in the usual manner. When *C. krusei* was added to products of the action of the enzyme on sucrose, namely glucose, fructose, sucrose, and I, II and III, only the monosaccharides disappeared; I was not attacked. These results are shown diagrammatically in Fig. 3.

As in the case of mould sucrase, the activity incorporated into the sucrose spot in the presence of radioactive fructose (Table 9) could be ascribed to the formation of a sugar (component *C*, Table 11) containing no glucose residues. This and a second sugar (component *D*) were separated, in a manner

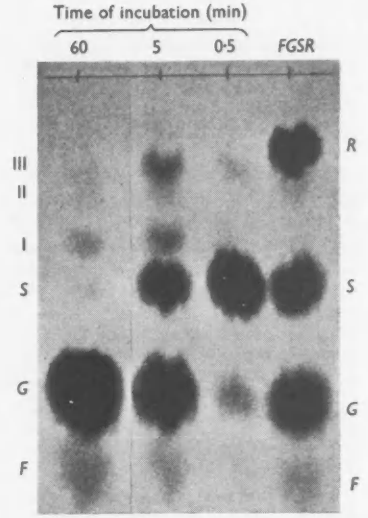

Fig. 2. The action of yeast sucrase on sucrose. Chromatogram of 2 μl. samples of a reaction mixture at 20° containing 400 mg. sucrose, 2·0 ml. 0·05 M sodium acetate buffer, pH 5·0, and 0·4 ml. 1% (v/v) yeast sucrase. I, probably a disaccharide; II and III, trisaccharides. I, II and III contain both glucose and fructose. Other symbols as in Fig. 1.

Table 7. *Some effects caused by the presence of added glucose or fructose on the action of yeast sucrase on sucrose*

The experiment was performed as described in Table 2, except that the enzyme solution used was 1% (v/v) yeast sucrase. Results are expressed as mg. combined fructose/ml.

		Time of incubation (min.)			
Substrates	Sugars estimated	0	20	40	90
Sucrose	Sucrose	49·5	24·8	10·3	1·4
	Oligosaccharides I	—	0·6	0·6	0·3
	II + III	—	3·6	2·8	0·8
Sucrose + glucose	Sucrose	51·0	28·0	15·1	3·0
	Oligosaccharides I	—	1·3	1·8	1·5
	II + III	—	2·6	3·4	1·7
Sucrose + fructose	Sucrose	50·5	29·8	16·6	5·0
	Oligosaccharides I	—	0·6	0·6	0·6
	II + III	—	2·8	3·1	1·5

Table 8. *The action of yeast sucrase on sucrose and radioactive glucose*

A. Total activities. The reaction mixture contained 40 mg. sucrose, 19·9 mg. radioactive glucose with total activity about 10 μc, 0·175 ml. 0·04 M sodium acetate buffer, pH 5·0, and 0·025 ml. yeast sucrase. Incubation was carried out in an Agla syringe at 20°. Samples (2 μl.) were delivered on to filter-paper sheets. Results are expressed as counts/min. in the sugar spot.

	Time of incubation (min.)				
After development	2	6	11	15	60
Glucose spot	5900	5820	5470	5720	6060
Sucrose spot	37	29	34	16	23
Spot I	183	227	152	113	68
Spot II	0	8	2	4	1
Spot III	12	11	17	8	5
Total	6132	6095	5675	5861	6157

B. Specific activities. Same reaction mixture as for *A*. Values deduced from paper chromatogram analyses combined with above table. Results are expressed as counts/min./μg. of free or combined glucose.

Glucose spot (initial specific activity 36)	—	23·5	20·5	—	18
Spot I*	—	25·4	25·3	—	—

* The glucose content of this material was deduced from its fructose analysis, assuming it to be a fructosyl-glucose disaccharide.

Table 9. *The action of yeast sucrase on sucrose and radioactive fructose*

A. Total activities. The reaction mixture contained 42 mg. sucrose, and 20 mg. radioactive fructose with total activity about 10 μc. Experiment performed as described in Table 8. Results are expressed as counts/min. in the sugar spot.

	Time of incubation (min.)				
	2	6	11	15	60
Before development (total applied to paper)	6757	6494	6888	6154	6910
After development					
Fructose spot	6228	5673	5882	5796	6555
Sucrose spot	76	134	147	119	57
Spot I	6	7	15	11	5
Spot II	8	14	12	11	5
Spot III	13	17	16	10	12
Total	6331	5845	6072	5947	6634

B. Specific activities. Same reaction mixture as for *A*. Values deduced from paper chromatogram analyses, combined with above table. Results are expressed as counts/min./μg. of free or combined fructose.

Frustose spot (initial specific activity 36)	—	23·3	21·4	—	18
Sucrose spot	—	2·0	5·4	—	—

Table 10. *The identity of the radioactive sugars incorporated into oligosaccharides synthesized by yeast sucrase*

Two-dimensional chromatograms were developed using samples from the experiments described in Tables 8 and 9. Sugars were hydrolysed after first development. No attempt at accurate quantitative estimation was made. Results are expressed as counts/min. in the sugar spot.

	Spots II and III	Spot I	Sucrose	Glucose	Fructose
Added radioactive glucose (10 min. sample)					
1st development	6	170	20	4920	−2
2nd development					
Derived glucose	2	156	14	4255	—
Derived fructose	−3	0	−3	5	−5
Added radioactive fructose (5 min. sample)					
1st development	2	16	94	—	4326
2nd development					
Derived glucose	1	8	−2	—	—
Derived fructose	6	14	72	—	3847

Table 11. *Investigation of the components giving rise to the 'sucrose' spot after incubation of yeast sucrase with a mixture of sucrose and radioactive fructose*

For description of the experiment see text. Components *C* and *D* were separated by gradient elution.

	Total fructose content (mg.) (*a*)	Total glucose content (mg.) (*b*)	Ratio of glucose: fructose, from (*a*) and (*b*) (*c*)	Counts/min. estimated from filter paper: Per μg. hexose (*d*)	Total, from (*a*) + (*b*) and (*d*) (*e*)	Fraction of total in mixture, from (*e*) (%)
Component *C*	2·6	0·01	0·00	16·7	$43·5 \times 10^3$	89
Component *D*	3·8	3·4	0·9	0·8	$5·7 \times 10^3$	11

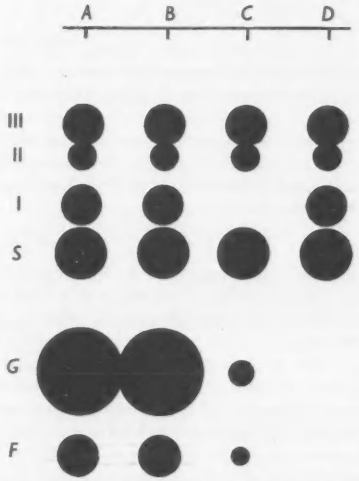

Fig. 3. The effect of *C. krusei* on the action of yeast sucrase on sucrose. Diagram of chromatogram. *A*, solution containing 2·0 ml. 3% (w/v) sucrose, 1·0 ml. H_2O, and 0·1 ml. 0·5% (v/v) yeast sucrase. *B*, as *A* but 1·0 ml. of a boiled suspension of *C. krusei* substituted for 1·0 ml. water. *C*, as *B* but with living *C. krusei* substituted for the boiled yeast. *D*, solution *A* boiled after 12 hr. incubation, cooled, and 1·0 ml. added to 1·0 ml. suspension of living *C. krusei*. Solutions incubated 12 hr. at 30°. Other symbols as in Figs. 1 and 2.

similar to that already described for the mould enzyme reaction, from a 1 hr. reaction mixture at 20° containing 50 mg. sucrose, 50 mg. radioactive fructose with total activity about 20 μc, 0·2 ml. 0·05 M sodium acetate buffer, pH 5·0, and 0·1 ml. 4% (v/v) yeast sucrase; the isolated sugars had R_F similar to sucrose. The results of the analyses are given in Table 11.

DISCUSSION

The experiments described in the previous section confirm the findings, first noted by Edelman (1950), Bacon & Edelman (1950), Blanchard & Albon (1950) and Bacon (1952), that sucrase preparations from both yeasts and moulds are capable of synthesizing oligo- and hetero-saccharides, apparently by the transfer of fructose residues from sucrose to suitable acceptors. Bealing & Bacon (1953) have suggested that all previously described invertase preparations from moulds have had similar transferring ability and Fischer *et al.* (1951), using electrophoretically purified yeast sucrase, found it capable of transferring fructose residues. Therefore, although little attempt was made to purify the enzyme preparations, it is considered justifiable to consider the results described here as of general application to mould and yeast sucrases. The term 'invertase' has come to be associated with a simple hydrolysis of sucrose to glucose and fructose; it is now suggested that the term 'sucrase' is more appropriate in view of the many types of reaction which these preparations are capable of catalysing using sucrose as substrate.

Sucrases have been classified into two major groups, viz. glucosaccharases (α-glucosidases, attacking sucrose at the glucose moiety) and fructosaccharases (β-fructofuranosidases, attacking at the fructose moiety). An account of the development of this classification has been given by Bealing & Bacon (1953); it is sufficient to say here that a major factor was the observation that some sucrases were inhibited by glucose and some by fructose. It has been generally agreed that the former group contained, among others, sucrases derived from moulds, and the latter group those derived from yeasts (e.g. see Neuberg & Mandl, 1950).

Inhibition by added glucose and fructose of sucrose breakdown certainly seems to occur (Tables 2 and 7), but the experiments described in this paper show that the effect of adding these sugars is more complex than was formerly believed and that, apart from any physical effect they may exert upon the enzyme, they enter into the reactions in characteristic ways. Although these experiments have indicated major differences between mould and yeast sucrases, they have further provided evidence that the previous classification of these into glucosaccharase and fructosaccharase is not

justified (cf. Bealing & Bacon, 1953; Bealing, 1953). They have also elucidated the hexose-transfer mechanisms involved in the formation of some of the oligosaccharides.

Radioactive-glucose experiments

A major difference between mould and yeast sucrases is demonstrated by the respective actions of these enzymes on mixtures of sucrose and radioactive glucose. Radioactivity was rapidly incorporated as combined glucose into oligosaccharides in each case.

With mould enzyme, sucrose was the first oligosaccharide to become labelled, indicating that the action responsible was a transfructosylation which may be considered to occur thus:

$$[G \rightarrow \leftarrow F] + \text{enzyme-H} \rightarrow \text{enzyme-F} + [G \rightarrow H]$$

$$\text{enzyme-F} + [{}^{*}G \rightarrow H] \rightarrow [{}^{*}G \rightarrow \leftarrow F] + \text{enzyme-H}.$$

Combining the equations we have

$$[G \rightarrow \leftarrow F] + [{}^{*}G \rightarrow H] \rightarrow [G \rightarrow H] + [{}^{*}G \rightarrow \leftarrow F],$$

where $[G \rightarrow \leftarrow F]$ denotes sucrose, and $[G \rightarrow H]$ glucose, the arrow indicating the reducing group and '*' indicating radioactivity. In these equations the fructosyl and not the fructoside group is represented as being transferred: there is some evidence that this alternative is correct (Koshland & Stein, 1953). Later in the reaction the saccharides of higher molecular weight (compounds α and β, Fig. 1) which are formed become labelled in their glucose components. This supports the theory that these compounds, known to have fructose:glucose ratios greater than unity and to be composed entirely of these two sugar residues, are formed by further fructose transfer, e.g.

$$\text{enzyme-F} + [G \rightarrow \leftarrow F] \rightarrow [G \rightarrow \leftarrow F \leftarrow F] + \text{enzyme-H},$$

a hydrogen atom on the fructoside moiety of sucrose being replaced by a fructosyl group (Fischer *et al.* 1951; Edelman & Bacon, 1951; Wallenfels & Bernt, 1952; Pazur, 1952; Bealing & Bacon, 1953). Table 3 shows this effect to some extent, but it is better demonstrated in Table 5. In the latter an enzyme preparation from *P. spinulosum* was used; this was known to have higher transferring capacity to sugar (as evidenced by oligosaccharide formation) relative to water (hydrolysis) than have preparations from *A. oryzae* (Bealing & Bacon, 1953). The structure of the trisaccharide α has recently been established as *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside (Bacon & Bell, 1953). The fructose-transferring capacity of mould sucrase has been used in this laboratory to synthesize glucose-labelled sucrose on a preparative scale from inactive sucrose and radioactive glucose (L. H. May, unpublished results). Spot *x* (Fig. 1, Tables 3 and 4) was possibly a diglucose formed by the condensation of two molecules of free glucose; it was formed only in very small amount.

Yeast sucrase did not catalyse the incorporation of free glucose into sucrose, but into a reducing oligosaccharide designated I (Table 8*A*). This was almost certainly a disaccharide in which a hydrogen atom from one of the non-reducing hydroxyl groups of a glucose molecule was exchanged for a fructosyl group. This indicates that the transfer in this case proceeds thus:

$$\text{enzyme-F} + [G \rightarrow H] \rightarrow [F \rightarrow G \rightarrow H] + \text{enzyme-H}.$$

The position of attachment of fructose to the glucose molecule is not known but, in view of the findings of Whelan & Jones (1953) who isolated a similar disaccharide from the products of the action of yeast sucrase on a mixture of *O*-β-D-methyl fructofuranoside and glucose, it is probably at $C_{(6)}$. The overall reaction was thus:

$$[G \rightarrow \leftarrow F] + [{}^{*}G \rightarrow H] \rightarrow [F \rightarrow {}^{*}G \rightarrow H] + [G \rightarrow H].$$

The absence of radioactivity in the reducing disaccharide when labelled fructose was added under comparable conditions (Table 9), shows that a fructose and not a glucose radical was transferred to give I. This explains the characteristically later appearance of this spot than spots II and III during the normal reaction of yeast sucrase with sucrose, and its earlier appearance and enhanced increase in the presence of added glucose at the start of the reaction (Table 7). The specific activity values given in section *B* of Table 8 provide further evidence supporting the suggested mechanism of formation of I. Thus the specific activity of the glucose moiety of I at any time should have been not less than that of the free glucose at the same instant. It could be expected to have been rather higher as the specific activity of the free glucose progressively decreased owing to the liberation of free glucose from sucrose.

Radioactive-fructose experiments

The addition of fructose to sucrase-sucrose mixtures had less effect upon the production of higher oligosaccharides than did that of glucose under similar conditions (Tables 2 and 7). It was noticeable, however, that there was considerable inhibition of sucrose disappearance as estimated by fructose content of the sucrose spot. Addition of labelled fructose showed that activity became incorporated into the sucrose spot under the influence of both types of enzyme (Tables 4 and 9). This active material was entirely hydrolysed by sucrase to give only inactive glucose and labelled fructose.

Fructose-labelled sucrose could not be formed by fructose transfer from sucrose under these conditions, and the possibilities remained that glucose transfer from sucrose to free fructose had occurred or that a compound other than sucrose was present in the sucrose spot. The following evidence indicates the latter alternative to be correct.

It was noticeable in the absence of added glucose or fructose that with either type of enzyme the sucrose spot appeared more yellow in colour (as shown by the benzidine/trichloroacetic acid spraying reagent) towards the end of the reaction than at the start. When free fructose was added to the reaction mixture this effect was greatly increased. These observations indicated that the fructose:glucose ratio became greater than unity as the reaction proceeded, and this was supported, in the case of the mould enzyme, by analytical results. It appeared, therefore, that compounds with a greater fructose content than sucrose but with the same R_F were being formed. By the method of gradient elution it was eventually found possible to separate two sugars with R_F similar to sucrose from the products of both the mould and yeast enzyme reactions on mixtures of sucrose and labelled fructose (see Tables 6 and 11). The two sugars emerged from the chromatographic column very close together and were possibly not completely separated. One of the sugars of each pair (component *A*, mould enzyme; component *C*, yeast enzyme) contained only fructose units, while the other (component *B*, mould enzyme; component *D*, yeast enzyme) contained equal proportions of fructose and glucose and was almost certainly sucrose. In both cases the glucose-free component contained about 90% of the total activity. Both components were hydrolysed by yeast sucrase entirely to monosaccharide.

From these results it seems very probable that the activity incorporated into the 'sucrose' spot in the presence of labelled fructose was due in each case to the formation of a fructose disaccharide with the same R_F (under the chromatographic conditions used) as sucrose. As neither mould nor yeast enzyme had any relevant effect upon a mixture of glucose and fructose, difructose formation is considered to take place by transfer of fructose from sucrose to free fructose (cf. Bealing, 1953).

Further support for this hypothesis was provided by the results of experiments with raffinose and free fructose, possible confusion of sucrose with other sugars of similar R_F thus being eliminated. Of the many compounds formed when using a preparation from *P. spinulosum*, one was produced in relatively small amount and was shown by two-dimensional chromatography to contain only fructose residues. Where labelled fructose was added this compound became radioactive. Since raffinose is known to act as fructose donor under the conditions of these experiments, this compound is considered to have been a difructose.

The specific activity values shown in Tables 3, 4 and 9 can be explained on the basis of difructose formation. As the quantity of material in the 'sucrose' spot was deduced from fructose analysis assuming a glucose:fructose ratio of unity, weight values were increasingly overestimated during the reaction owing to the progressive formation of difructose. Thus the specific activities attributed to sucrose in Table 3 (labelled-glucose experiment) decreased towards the end of the reaction, and were probably underestimated at all stages. The true value for glucose combined in sucrose or other saccharides would be expected to approach that of the free glucose and would eventually be in equilibrium with it. Compound α did reach a high value, as a true estimate of the glucose content was probably obtained here (glucose:fructose ratio = 0·5).

Fructose turnover

The true capacity of a sucrase for the turnover of fructose radicals can be estimated only when the rates of all the individual reactions which constitute its action on sucrose are fully known. These include transfer from sucrose to the elements of water (hydrolysis), to liberated glucose and fructose, to sucrose, and to oligosaccharides so formed, some or all of which may act as acceptors; in addition, these oligosaccharides may act as fructose donors. It is virtually certain that the rates of these reactions vary widely. An estimate of fructose transfer can be obtained, however, from experiments of the type already described using radioactive sugars. In the experiment described in Table 3, in which the reaction mixture contained about 0·5 M sucrose and 0·5 M glucose, total transfer per ml. of the reaction mixture in the first 30 min. (during which time the sucrose concentration fell only by about 8% of its initial value) was made up of transfer to (*a*) the elements of water, 2·5 mg. by direct fructose estimation, (*b*) free glucose, 2·4 mg. from total radioactivity incorporated into sucrose divided by the mean specific activity of free glucose during this period and (*c*) sucrose, 0·7 mg. by estimation of fructose in spot α (glucose:fructose ratio = 0·5). All these values are necessarily approximate. Assuming that transfer to free fructose, and from difructose, compound α, and labelled sucrose was negligible during this period, the total transfer was about 5·6 mg. fructose/ml.; of this, about 45% appeared free and 13% was incorporated into oligosaccharide of greater molecular weight than sucrose.

In the case of added radioactive fructose (experiment described in Table 4, about 0·5 M sucrose and 0·5 M fructose) the corresponding fructose-transfer values per ml. for the first 30 min. (sucrose concentration reduced by about 12% of its initial value)

were (*a*) to the elements of water, 3·0 mg., (*b*) to free fructose, 0·7 mg. and (*c*) to sucrose, 2·7 mg. The total transfer was thus about 6·4 mg./ml., of which 47% appeared as free fructose and 42% formed oligosaccharide. There may be a larger error here as it was not possible to estimate transfer to the free glucose liberated during the reaction. Although the concentration of glucose was low (about 0·03M at 30 min.), its affinity for the enzyme is unknown.

Although the total transfer values in both cases are of roughly the same order, it does appear that the presence of free glucose may cause some inhibition as compared to the presence of free fructose. There is very marked competition for transferred fructose between glucose and sucrose, this being for very different groups on the respective molecules, as the glucose accepts on its reducing group, whereas sucrose accepts on position 1 of the fructose moiety. It is interesting to note that the formation of levan from sucrose by levan sucrase is also competitively inhibited by free glucose (Hestrin & Avineri-Shapiro, 1944).

It is obvious from considerations mentioned above that liberation of free reducing sugar may give no indication of the total capacity of a sucrase to transfer fructose residues.

General considerations

The transfer of fructose radicals which has been discussed above is presumably effected by means of an intermediary fructose–enzyme complex; from the evidence it appears that the enzymes are incapable of catalysing glucose transfer (contrast the findings of White & Maher (1953), who used honey sucrase). Both types of enzyme may therefore be considered to be β-fructofuranosidases in that the fructofuranose group is more intimately involved with the enzyme during the cleavage of sucrose. In common with other workers it is shown that several sugars can act as fructose acceptors; the compounds formed, however, may differ according to the enzyme used.

It was considered possible that a particular case of the general reaction might be the enzymic net synthesis of sucrose by transfer of fructosyl radicals from donors other than sucrose to glucose under the influence of mould sucrase. However, when 'Takadiastase' was allowed to act upon mixtures of glucose and inulin or *O*-β-D-methyl fructofuranoside (prepared by the method of Bacon, 1952), although free fructose was liberated, there was no indication of sucrose or oligosaccharide synthesis. This failure could be attributed to substrate specificity (implying that fructose liberation and transfer are due to different enzymes under these conditions), or to a high breakdown:synthesis ratio of oligosaccharides, or to thermodynamic considerations. Should the standard free energy of hydrolysis of sucrose be considerably greater than that of the fructoside, the number of sucrose linkages formed would be very small; under the conditions used this could be increased only by effective coupling between hydrolysis and fructose transfer to glucose.

The significance of fructose transfer in metabolism is as yet unknown. There appears to be correlation between the mechanisms described here and the wide occurrence in the plant kingdom of fructose polymers ranging from sucrose, through oligosaccharides, to polyfructoses (Bacon & Edelman, 1951; Porter & Edelman, 1952). Fructose-transferring enzymes do exist in plants, e.g. Jerusalem artichoke (Edelman & Bacon, 1951), and barley (Edelman, unpublished experiments). Similar glycosidic linkages are present in the naturally occurring plant fructosans and in products of mould and yeast sucrases; thus the 2:6-fructofuranoside linkage is found in kestose (spot III of the yeast reaction, see Albon, Bell, Blanchard, Gross & Rundell, 1953) and in the levan series of fructose polymers (Challinor, Haworth & Hirst, 1934; Laidlaw & Reid, 1951), while the 2:1 linkage occurs in spot α of the mould reaction (Bacon & Bell, 1953) and in the inulin series (Hirst, McGilvray & Percival, 1950; Bacon & Edelman, 1951).

SUMMARY

1. Free glucose and fructose enter into the action of mould and yeast sucrases on sucrose in characteristic ways. Previous reports of inhibition of these enzymes by free hexose may be partly or wholly explained on this basis.

2. The use of radioactive sugars showed that, under the influence of mould sucrases, free glucose and the glucose combined in sucrose are exchangeable by means of the transfer of fructose residues. In this way glucose-labelled sucrose has been obtained. Further fructose transfer gives rise to glucose-labelled saccharides of greater molecular weight.

3. Similar experiments have shown that yeast sucrase will transfer fructose residues from sucrose to free glucose to form a reducing compound, probably a fructosyl-glucose disaccharide.

4. Both types of enzyme transfer fructose residues from sucrose to free fructose to give a glucose-free sugar, probably a difructose.

5. Mould and yeast sucrases are both β-fructofuranosidases but differ in their specificity towards fructose acceptors.

6. The capacity of sucrases for fructose turnover cannot be deduced from hexose liberation.

7. Some correlation between fructose transfer and naturally occurring fructose polymers has been drawn.

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Chromatographic Determination of Cystine as Cysteic Acid

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The determination of the cystine content of proteins is frequently complicated by the instability of cystine and cysteine during protein hydrolysis, particularly in the presence of carbohydrate (cf. Martin & Synge, 1945; Block & Bolling, 1951). Oxidation of the cystine and cysteine residues in the intact protein can convert the amino acids into cysteic acid, which is stable under the conditions of acid hydrolysis. The present method is based upon this step, followed by the quantitative determination of cysteic acid by ion-exchange chromatography on columns of Dowex-2. The chromatographic identification of cysteic acid as the end product of the oxidation contributes to the specificity of the method. The procedure does not distinguish between cystine and cysteine residues in the protein.

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The conditions of Toennies & Homiller (1942) for the oxidation of the sulphur-containing amino acids by performic acid have been applied to proteins in several investigations. Toennies (1942) oxidized casein to convert methionine into the sulphone and observed the disappearance of part of the cystine and all of the tryptophan. Sanger (1949) used the reaction to split the —S—S— bridges of insulin as a first step in the investigation of the structure of the hormone. The reaction has been applied to oxytocin by Mueller, Pierce, Davoll & du Vigneaud (1951). The oxidation of wool by performic acid has been studied by Blackburn & Lowther (1951).

A quantitative study of the yield from the reaction requires an accurate method for the determination of cysteic acid in protein hydrolysates. Paper chromatograms have been used qualitatively to identify cysteic acid after its formation from

cystine (Dent, 1948). Consden, Gordon & Martin (1948), Consden & Gordon (1950), and Sanger & Tuppy (1951) have used a weakly basic ion-exchange resin (IR-4B) to separate cysteic acid and cysteic acid peptides. The quantitative method of ion-exchange chromatography developed for the present study is based upon the principles and techniques used in the determination of amino acids on sulphonated polystyrene resins (Moore & Stein, 1951), but uses a strongly basic resin under conditions similar to those employed with Dowex-1 and Dowex-2 in the chromatography of nucleotides by Cohn (1950) and of acids of the citric acid cycle by Busch, Hurlbert & Potter (1952).

Cysteic acid may also be determined on Dowex-50 columns, through which it passes without measurable retardation (Moore & Stein, 1951). Its determination under these conditions would be reliable only if one were certain that the hydrolysate contained no other strongly acidic, ninhydrin-positive constituents. The use of Dowex-2, with hydrochloric or chloroacetic acid as eluent, provides preferable resolving power in a system in which all of the common amino acids, including taurine and aspartic and glutamic acids, emerge first, with cysteic acid and its homologues having distribution coefficients strongly in favour of the resin phase. Mixtures of homocysteic and cysteic acids, for example, are resolved under the conditions of the present experiments, with the former emerging as a peak just preceding that of cysteic acid.

PROCEDURE

Oxidation. The performic acid reagent was prepared by the addition of 1 vol. of 30% (w/w) H_2O_2 to 9 vol. of 88% (w/w) formic acid. The solution was allowed to stand for 1 hr. at room temp. to permit the performic acid concentration to reach the maximal value (Toennies & Homiller, 1942). 25 ml. of the reagent, previously cooled to 0°, were added to the sample to be oxidized (preferably taken to contain 1–2 mg. of cystine). In the oxidations of cystine the amino acid was dissolved in formic acid (20 mg. in 10 ml.) and 1 ml. portions were added to the performic acid solution. With amino acids and soluble proteins the oxidation was allowed to proceed at 0° for 4 hr. With insoluble samples the ice-water bath was placed in a refrigerator overnight (16 hr.). At the end of the reaction time most of the reagent was removed under reduced pressure (water pump) at bath temp. 30–40° on a rotary evaporator of the type described by Craig, Gregory & Hausmann (1950). The concentration was stopped as soon as the bulk of the reagent was removed, yielding a syrupy ribbon on the wall of the rotating flask. This stage was reached in 10–15 min. Attempts to carry the evaporation to dryness under these conditions may in some instances lead to low recoveries. (If desired, the residue from the concentration on the water pump can be taken to dryness without loss of cysteic acid by placing the flask for 1–2 min. on a high-vacuum line (oil pump) equipped with a solid-CO_2 trap.)

If the experiment was a control oxidation on cystine, the syrupy residue was taken up in water, transferred to a 10 ml. flask and a 1 ml. portion of the slightly acid solution was added to the Dowex-2 column.

Hydrolysis. In the analysis of proteins, the residue from the oxidation was immediately dissolved or suspended in 50 ml. of 6 N-HCl and boiled under reflux for 20 hr. It is not necessary to remove the last traces of reagent before the addition of the HCl. If the initial sample contained more than 90% of carbohydrate, 100 ml. of HCl were used for hydrolysis. The oxidation, evaporation and hydrolysis were conveniently carried out in the same 250 ml. ground-joint flask to avoid transfers. The hydrolysate was filtered through sintered glass to remove humin. The filtrate was taken to dryness on a rotary evaporator. After the flask had been placed over NaOH in a vacuum desiccator for a few hours, the residue was taken up in water and transferred to a 10 ml. volumetric flask. Before making the sample to volume the pH was adjusted to about 5 by drops of 0·1 N-NaOH, using methyl red as internal indicator.

Chromatography. From the treated hydrolysate, a 1 or 2 ml. portion (corresponding to 0·1–0·2 mg. cystine) was added to a 0·9 × 15 cm. column of the chloroacetate form of 200–400 mesh/in. Dowex-2-X 10 (Dow Chemical Co., Midland, Mich.). Cysteic acid was eluted with 0·1 N chloroacetic acid (Fig. 1). A forerun of 100 ml. of 0·01 N acid was used in the case of samples of very low cystine content (e.g. casein) or high carbohydrate content (Fig. 2). The effluent

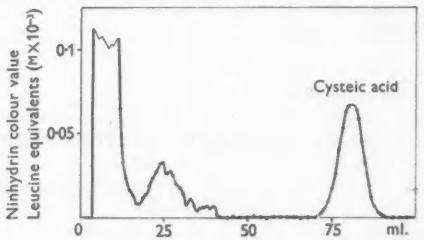

Fig. 1. Chromatography of a hydrolysate of oxidized Bence-Jones protein. The sample on the column corresponded to 3·9 mg. of protein (79 μg. cystine). Column, Dowex-2 (0·9 × 15 cm.). Eluent, 0·1 N chloroacetic acid.

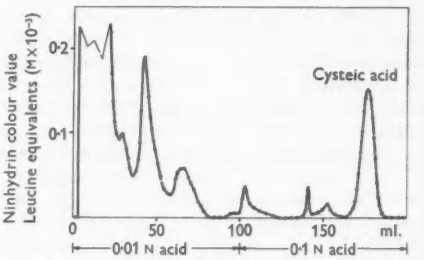

Fig. 2. Chromatography of a hydrolysate of oxidized cow's milk. The sample on the column corresponded to 0·5 ml. of milk containing about 0·2 mg. cystine. Column, Dowex-2 (0·9 × 15 cm.). Eluent, aqueous chloroacetic acid.

was collected in 1 ml. fractions at 5 ml./hr. and analysed by the photometric ninhydrin method (Moore & Stein, 1948) as employed in this laboratory (Schram, Dustin, Moore & Bigwood, 1953), using 1 ml. of reagent for each fraction. All eluents contained 1% (v/v) of 33·3% (w/w) aqueous BRIJ 35 (an ether of polyethylene glycol made by Atlas Powder Co., Wilmington, Del.) as detergent (as recommended by Moore & Stein, 1951). The 0·1 N chloroacetic acid fractions were neutralized with 0·1 ml. of 1·25 N-NaOH. The colour yield of cysteic acid (anhydrous) was 1·01 relative to leucine. Since the accuracy of the method is dependent upon the exact colour yield, the factor should be determined under the user's experimental conditions of sample size and neutralization. The yield in the oxidation of cystine should also be determined as a check on the procedure.

The Dowex-2 resin was washed between experiments with 50 ml. N chloroacetic acid, followed by equilibration with the strength of acid to be used for the chromatography. The resin was initially prepared for use by washing the hydrochloride form thoroughly with N-HCl and passing through N sodium chloroacetate until the effluent was free of chloride (cf. Groth, Mueller & LePage, 1952).

RESULTS AND DISCUSSION

The yield in the oxidation. The chromatographic technique permitted a detailed study of the yield of cysteic acid obtained from cystine in the performic acid oxidation. Under the prescribed conditions both cystine and cysteine gave 90 ± 2% of the theoretical yield of cysteic acid (Table 1). In the application of the method to proteins the quantities of cysteic acid measured in the hydrolysates have routinely been divided by 0·90 to give the final percentage figures (Table 2). The justification for the use of the correction rests on the observation that the factor is applicable to cystine and cysteine, to *N*-substituted cystine derivatives (*NN'-bis*-chloroacetylcystine monohydrate, Table 1), and to proteins of established cystine content (e.g. bovine serum albumin, Table 2). A reaction which would give a 100% yield in the oxidation would be preferable, but the potential advantages of the cysteic acid approach indicate that with a 90% yield the method can serve the practical purposes for which it has been designed.

The variables in the process were systematically investigated. The yield of cysteic acid was not altered by increasing the concentration of hydrogen peroxide in the formic acid, by lengthening the time of reaction, by oxidation at −10°, or by removal of the reagent by freeze-drying. The choice of 0° was based upon the observation that the yield from cysteine was several per cent lower than the recovery from cystine when the oxidation took place at room temperature. The data in Table 1 demonstrate that the chromatographic procedure gives 100% recovery when cysteic acid is the starting material and that there is not more than 3% loss when cysteic acid is put through both the oxidation and hydrolysis steps of the procedure. Thus there appear to be side reactions in the oxidation leading to 10% of products other than cysteic acid.

Table 1. *Yields of cysteic acid in control experiments*

Starting material	Treatment before chromatography	Cysteic acid (% recovery or yield)	
L-Cysteic acid	None	99	
		99	
		100	
		99	
	'Hydrolysis' in 6N-HCl	99	
	'Oxidation' by performic acid	97·5	
		96	
		97	
	'Oxidation' and 'hydrolysis'	97	
	'Oxidation' at +20°	97	
L-Cysteine	Oxidation and 'hydrolysis'	89	Av. 90 ± 2
		90	
		89	
L-Cystine	Oxidation and 'hydrolysis'	89	
		88	
		92	
NN'-bis-Chloroacetyl-L-cystine	Oxidation and hydrolysis	88	
		90	
L-Cystine	Oxidation at −10°	92	
	Oxidation at +20°	87	
	Oxidation in 44% formic acid instead of 88%	84	

Table 2. *Application of the method to proteins*

Serum albumin and casein were dried (20 mm. Hg) at 100° over P_2O_5. Hair was dried in air at 100° to constant wt., having been first de-fatted in ether (Soxhlet). Total nitrogen content is only given when actually determined in the same sample. This was not considered necessary in the case of Armour's 'Bovalbumin' and of human hair.

			Cystine			Literature values	
Material analysed	Treatment	Total N (%)	g./100 g.*		N as % of total N	g./100 g.	N as % of total N
50 mg. serum albumin, bovine (Armour)	Standard	—	6·28		—	—	—
		—	6·07	Av. 6·34	—	—	—
Same +1 g. starch	Standard	—	6·43		—	—	—
50 mg. serum albumin	6N-HCl at 40° for 5 days before oxidation	—	6·50		—	6·52†	—
		—	6·42		—	—	—
50 mg. serum albumin	Oxidation at +20°	—	5·89		—	—	—
500 mg. casein‡	Standard (chromatogram started with 0·01N acid)	14·5	0·34		0·27	—	—
		—	0·33		0·27	—	—
375 mg. casein‡	Oxidation for 16 hr.	14·5	0·33		0·27	Approx. 0·3‖	—
20 mg. human hair (5·38% S)§	Oxidation for 16 hr.	—	18·0		—	—	—
		—	18·1		—	14–18‖	—
1 g. beef	Sample not ground (Oxid. 16 hr.)	3·50	0·28		0·93	—	Approx. 0·95‖
	Sample ground with sand (Oxid. 16 hr.)	—	0·27		0·90	—	—
1 g. veal	Sample not ground (Oxid. 16 hr.)	3·53	0·28		0·92	—	—
		—	0·28		0·92	—	—
1 g. barley¶	Ground whole meal (Oxid. 4 hr.)	1·53	0·22		1·7	—	Approx. 0·6**
		—	0·24		1·8	—	—
			0·24		1·8	—	—
			0·23		1·8	—	—

* On basis of dry wt. for serum albumin, casein and hair; on basis of whole foods of specified N content for beef, veal and barley.
† Brand, Kassel & Saidel (1944) (cystine + cysteine).
‡ Corrected for moisture (8·25%) but not for ash.
§ The cystine found accounts for 89% of the total sulphur.
‖ Cf. Block & Bolling (1951).
¶ Dustin, Schram, Moore & Bigwood (1953).
** Ågren (1949).

Application to proteins. The rate of oxidation of cystine residues in proteins appears to be similar to that of the free amino acid. Bovine serum albumin gave the same yield of cysteic acid at 2, 4, 8 and 20 hr. of oxidation. Neither heat denaturation nor prior partial hydrolysis of the protein altered the yield (Table 2). The recovery was not affected by the presence of carbohydrate to the extent of 20 times the weight of the serum albumin. The reaction was effective on insoluble proteins; human hair or cubes of beef were fully oxidized after standing in the reagent overnight (Table 2). Stirring of the reaction mixture was not necessary. When samples of milk were oxidized, 5 ml. portions were concentrated to about 2 ml. in the flask before the addition of the 25 ml. of chilled reagent.

The need for the use of the 0·01N chloroacetic acid forerun is determined by whether or not the effluent curve with 0·1N acid falls to the base-line before the emergence of the cysteic acid peak. In the analysis of foods of high carbohydrate content, part of the possible interference in the early portion of the effluent curve comes from the reddish colour yielded by the reaction of ninhydrin with carbohydrate decomposition products (Dustin, Czajkowska, Moore & Bigwood, 1953). In the developmental studies on the present method 0·010N- and 0·015N-HCl were used as the eluents with the chloride form of Dowex-2, under which conditions the rate of travel of cysteic acid is about the same as that obtained with the higher molarities of chloroacetic acid. In preparative chromatography the use of 0·015N-HCl would be preferable for the isolation of cysteic acid and cysteic acid peptides. In general, 0·1N reagents have the advantage of rendering the column relatively insensitive to any buffering action of the sample under analysis, and therefore give more reproducible results.

In addition to the applications referred to in Table 2, the method has been employed for the determination of cystine levels in a number of foods including human milk (Soupart, Moore & Bigwood, 1953) and cassava flour (Close, Adriaens, Moore & Bigwood, 1953).

SUMMARY

1. A method for the determination of the cystine plus cysteine content of proteins has been based upon oxidation of the protein by performic acid, hydrolysis by hydrochloric acid and chromatographic determination of the resulting cysteic acid on columns of a basic ion-exchange resin (Dowex-2).

2. The method can be used directly on products in which a high carbohydrate content may render other methods inapplicable, or in cases where the uncertainties which apply to the stability of cysteine and cystine even under favourable hydrolytic conditions may render an alternative method desirable. The procedure has been applied to the analysis of foods and purified proteins.

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Active Transport of Ions by Sub-Cellular Particles

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The biochemical aspects of active transport in a variety of cells have been studied intensively over the last few years (see reviews by Ussing, 1949, 1952; Davies, 1951), and it has now become clear that most secretory processes require supplies of energy from aerobic metabolism. The metabolic behaviour of a variety of sub-cellular particles has also been investigated, and this work has shown that the mitochondria are the site of nearly all the respiratory activity and oxidative phosphorylation of the cells that have been investigated (see reviews by Green, 1951; Schneider, 1953). Since experiments with 2:4-dinitrophenol indicate that oxidative phosphorylation is closely linked with active transport (Mudge, 1951; Krebs, Eggleston & Terner, 1951; Davies, 1951; Hodgkin & Keynes, 1953; Whittam & Davies, 1953), it seemed reasonable to suppose that mitochondria are closely associated energetically with secretory activity in cells. The mitochondria may also be the special structures responsible for the appearance of secretions at one particular wall of a cell. It therefore seemed worth while to investigate the possibility of a direct association between mitochondria and active transport.

Harman (1950) failed to find a concentration gradient of potassium between mitochondria and the surrounding medium. However, the particles he had isolated had been kept for more than 20 min. at 0° and this may have so reduced their metabolism that effects attributable to active transport could no longer be observed. This paper describes experiments in which preparations of 'cyclophorase' and mitochondria from sheep kidney cortex were

separated from the medium while they were actively metabolizing, and shows that these preparations are able to use metabolic energy to accumulate a wide variety of anions and cations to higher concentrations than in the medium.

Part of this work was communicated to the Biochemical Society on 19 September 1952 (Bartley & Davies, 1952). Similar results for sodium, potassium and water were obtained with liver mitochondria simultaneously and independently by Macfarlane & Spencer (1953). After this paper was written, the results of Stanbury & Mudge (1953) became available. They found that potassium could be retained by liver mitochondria against concentration gradients, and that it was only slowly exchangeable under their experimental conditions, which were widely different from those described here.

EXPERIMENTAL

Tissue preparations. Most of the experiments described were carried out with sub-cellular particles from sheep kidney cortex. The sheep was killed and the kidney removed within 3 min. and plunged into a mixture of frozen and liquid 0·9 % (w/v) KCl. It was used within 30 min. and kept at 0° during all stages of the preparation.

The 500 ml. glass vessel of a Waring Blendor containing 200 ml. of 0·9 % (w/v) KCl and 1·0 ml. of 2 N-NaOH was put in a deep freeze at −18° until ice began to form. The vessel was removed, 50 g. of the kidney cortex were added and the mixture was homogenized for between 1 min. 45 sec. and 2 min. This procedure produced the unfractionated suspension.

The homogenate was poured into four 50 ml. polythene tubes and centrifuged at 2000 *g* for 15 min. in an International Portable Refrigerated Centrifuge Model PR-1 with the temperature control set at −5°. The supernatant was poured off, together with a little of the sediment (fluffy microsomal layer). To the residue in each of the tubes were added about 5 ml. of the ice-cold washing fluid (0·9 % (w/v) KCl or 0·9 % (w/v) NaCl). After a few seconds stirring with a mechanically impelled glass paddle, a further 30 ml. of washing fluid were added and the mixture was again stirred (the addition of the washing fluid in two portions was to maintain a smooth suspension). The mixture was recentrifuged at 2000 *g* for 7 min. and gave the once-washed residue. The procedure of washing and centrifugation was repeated and after this washing the buff top layers of the sediment were removed and suspended in ice-cold 0·9 % (w/v) KCl and stored at 0°. Microscopic examination showed the particles to be largely mitochondria and nuclei. The whitish residue, which adhered tightly to the centrifuge tube and was rejected, consisted mainly of connective tissue, cell debris and erythrocytes. The mixture of nuclei and mitochondria is called 'cyclophorase' (Green, Loomis & Auerbach, 1948).

For the preparation of mitochondria, 25 g. of kidney cortex were put through a Latapie mincer and homogenized at 0° with a mixture of 70 ml. ice-cold 0·25 M sucrose and 1 ml. N-NaOH in a stainless steel homogenizer of the Potter-Elvehjem type, which was run for 1–2 min. at approx. 1000 rev./min. The resultant mixture was centrifuged at 700 *g* for 10 min. at 0°. The sediment was discarded and 8 ml. of 1·5 N-KCl were added to the supernatant. The mixture was thoroughly stirred and centrifuged at 2000 *g* for 15 min. giving unwashed mitochondria which were washed by the procedures described above. Rat heart-muscle sarcosomes were prepared according to Cleland & Slater (1953).

Media. In most experiments 2 ml. of the suspension (about 70 mg. dry wt.) were added to 2 ml. of a solution containing potassium phosphate buffer, pH 7·4 (0·02 M), sodium adenosine triphosphate (ATP) (0·002 M to 0·005 M) and $MgCl_2$ (0·002 M) and, usually, the oxidizable substrate, the final concentration of which is given in the description of the individual experiments.

Special chemicals. The preparations of ATP and α-oxoglutarate were those used previously (Bartley, 1954). [β:γ-$^{32}P_2$] ATP was prepared by the method of Hems & Bartley (1953). Pure sodium pyruvate was conveniently prepared by the following modification of Robertson's (1942) method: to 10 g. of pyruvic acid (twice fractionally distilled immediately before use) were added 15 ml. of water, and, in small amounts with constant stirring, 9·6 g. $NaHCO_3$. When gas evolution had ceased, the small amount of undissolved $NaHCO_3$ was removed by filtration and an equal volume of absolute ethanol was added while stirring. The voluminous, white, crystalline precipitate was filtered off, washed successively with 50 ml. absolute ethanol and ether, and dried over P_2O_5. The dry material was quite stable and was more than 99 % pure when analysed by the carboxylase method (Krebs & Johnson, 1937); it contained a trace of bicarbonate.

All chemicals, except sodium fumarate (British Drug Houses Ltd.), were of 'AnalaR' standard.

Incubation, isolation and extraction of particles. The aim of the experiments was to determine the concentrations of solutes in the particles and the medium separately. This necessitated separation by centrifugation, filtration having proved unsatisfactory. In designing the technique of centrifugation it was kept in mind that the failure of Harman (1950) to demonstrate concentration gradients between mitochondria and medium could have been due to the disappearance of gradients during the process of separation. Harman centrifuged at 0°; i.e. under conditions where energy supply is low. To avoid this complication the tissue suspension was centrifuged as quickly as possible at 20° after saturation of the suspension with O_2.

According to Ogston (1952) the time required for complete sedimentation for a given set of conditions is given by the formula, $\int_0^t \omega^2 dt = \text{constant}$, where ω is the angular velocity of the centrifuge head. The value of the constant was determined by observing the sedimentation times at low speeds; it showed that under our conditions complete sedimentation of the particles occurred when an acceleration of 24000 *g* was developed in 14 sec. and maintained for a further 10 sec. in the high-speed head no. 295 of an International Portable Refrigerated Centrifuge Model PR-1.

The procedure finally adopted for most groups of measurements was as follows: the media were pipetted into Pyrex centrifuge tubes (1·2 cm. internal diameter, 5·7 cm. long) in those experiments when the incubation period was up to 6 min., and into conventional Warburg vessels for longer experiments necessitating shaking. The medium (2 ml.) in the centrifuge tubes was gassed with O_2 at 0°, stoppered

and stored at 0° until ready for use. If measurements of radioactivity were to be made, the isotope was added at this stage. The media and a sample of the mitochondrial suspension were warmed to room temp. (20°); at zero time the centrifuge tube intended for the longest time of incubation was unstoppered and 2 ml. of the tissue suspension were blown into the medium from a pipette. The mixture was stirred with a portable micro-stirrer, and then allowed to stand at room temp. in the centrifuge head for the requisite period. (The stirrer was made by mounting a 'Minilec Model Motor' in a hand-torch case containing a 3 v dry cell, and attaching a stainless steel propeller to the motor spindle.) Successive tubes were treated in the same way until the last tube requiring the minimum time of incubation was reached. The suspension was added to this tube while in position in the head of the centrifuge. This was started at once and the speed was increased rapidly so that 24000 *g* was attained in about 14 sec. and maintained for exactly 30 sec. The centrifuge was then switched off; it stopped after a total time of spinning of 114 sec. The clear supernatants were quickly decanted from the centrifuge tubes into 5 ml. measuring tubes and both sets of tubes were then immediately plunged into a mixture of ethanol and solid CO_2. The contents were frozen within 3 sec. and 0·5 ml. of 30% (w/v) trichloroacetic acid (TCA) was added to each tube. These were singly warmed to room temp. and weighed; the volume of the fluid in the measuring tube was also recorded. After thorough stirring, and centrifugation, the supernatant was used for analysis. The completeness of extraction of ATP, inorganic phosphate, sodium and potassium was proved by re-extraction of the sediment with more TCA solution, by recovery of the added substance from the particles both before and after treatment with TCA, and in the case of sodium and potassium by dissolving the material in concentrated HNO_3 (Davies, Davies, Francis & Whittam, 1952). The pH was measured within a few seconds of separating the two phases without freezing the material.

Analytical methods. All concentrations given in this paper for the sub-cellular particles refer to the centrifuged material without allowance for adherent fluid. The pH was measured with the Beckman Probe Assembly, type 14400 and a Beckman pH Meter, model G. Sodium and potassium were measured with a flame-photometer by the method of Domingo & Klyne (1949) as modified by Terner, Eggleston & Krebs (1950). Each reading of an experimental solution was preceded and followed by a reading of a standard solution and at least four readings were taken of each sample in all cases. The standard error of the mean values for each sample was about 1%. Magnesium was measured by the method of Garner (1946). All compounds containing phosphorus were separated by paper chromatography (Eggleston & Hems, 1952; Krebs & Hems, 1953) and estimated after wet-ashing (Hanes & Isherwood, 1949), by the method of Berenblum & Chain (1938) as modified by Bartley (1953*b*).

Pyruvate, α-oxoglutarate and oxaloacetate were estimated as the 2:4-dinitrophenyl hydrazones using the 'specific extraction procedure' of Friedemann & Haugen (1943). Fumarate was measured as described by Krebs, Smyth & Evans (1940) and citrate by the method of Weil-Malherbe & Bone (1949).

O_2 uptake was measured manometrically in O_2 at 0° or 20° in the Warburg apparatus. A stick of yellow phosphorus was placed in the centre well throughout experiments in anaerobic conditions.

Measurements of radioactivity were made in a liquid counter with corrections for decay, background and dead time. Sodium and potassium were counted directly on the TCA extracts and phosphorus after wet-ashing paper strips from chromatograms (Bartley, 1953*b*). The changes in distribution of ^{32}P were used to calculate phosphorylation quotients (P/O ratios) by the formula of Krebs, Ruffo, Johnson, Eggleston & Hems (1953).

Contamination of sediments with supernatants. Even after centrifugation to constant volume, the sediment contained 'extraparticulate' fluid, owing to imperfect packing. The 'inulin space' (Gaudino, Schwartz & Levitt, 1948) was taken to represent the 'extraparticulate' fluid.

The inulin space of an actively metabolizing 'cyclophorase' suspension was measured as follows: 25 ml. of a fresh, twice-washed 'cyclophorase' preparation diluted with 3 vol. of 0·9% (w/v) KCl were added to 25 ml. of the medium containing 625 μmoles of α-oxoglutarate. O_2 was bubbled through the mixture for 10 min. at 20°, and two 20 ml. samples were then centrifuged at room temp. After decanting the supernatants, the inner surfaces of the centrifuge tubes were dried with filter paper and to each tube 1·0 ml. of fresh oxygenated medium containing 0·1% (w/v) 'purified inulin' (Thomas Kerfoot and Co. Ltd.) was added. The contents of the tubes were rapidly and thoroughly mixed and re-centrifuged. The supernatants were poured off and stored, the residues weighed, dried at 110° and reweighed. TCA (0·8 ml. of 30% w/v) was added to 0·8 ml. samples of supernatants and inulin-containing medium. The TCA extracts were centrifuged to remove precipitated protein and the inulin contents of measured samples estimated by the method of Bacon & Bell (1948). The inulin space was calculated from the dilution of the original inulin-containing medium.

RESULTS

Investigations with sheep kidney-cortex 'cyclophorase' preparations and unfractionated suspensions

Much information was available (Bartley, 1953*a*, *b*; 1954) about the metabolic behaviour of the kidney 'cyclophorase' preparations which consisted largely of mitochondria and nuclei, so this material was used in the early experiments on the maintenance and development of ionic gradients in sub-cellular particles from sheep kidney cortex.

Ionic gradients at 0°. 'Cyclophorase' preparations washed twice with 0·9% (w/v) potassium chloride solution and isolated at 0° were able to retain for several hours small amounts of sodium and potassium against adverse concentration gradients, but this ability was slowly lost on storing for several days at 2° (Table 1).

Ionic gradients at 20°. If the washed material isolated at 0° was warmed to 20°, this ability to retain sodium and potassium was rapidly lost. However, immediately after being warmed to 20° in the presence of ATP, magnesium and phosphate, both 'cyclophorase' preparations and the unfractionated suspension usually had higher concentrations of sodium and potassium than the

Table 1. *The sodium and potassium content at 0° of some unfractionated suspensions and 'cyclophorase' preparations from sheep kidney cortex*

The 'cyclophorase' preparations were twice washed in 0·9% KCl solution at 0°, and were prepared without prior storage of the kidney. The concentrations in the particles refer to the centrifuged material.

Expt. no.	Type of preparation and treatment	Content of sodium in (m-moles/kg.) Particles	Medium	Ratio of sodium in: particles / medium	Content of potassium in (m-moles/kg.) Particles	Medium	Ratio of potassium in: particles / medium
1	Unfractionated suspension	24·5	21·8	1·12	89	95·5	0·93
2	'Cyclophorase'	13·1	0·69	19·0	148·5	123	1·21
3	'Cyclophorase'; stored at 2° for 10 days	1·37	1·49	0·92	—	—	—
4	'Cyclophorase'; final suspension in 20 mM-$NaHCO_3$ in 100 mM-KCl	24·7	21·8	1·13	116·5	99·7	1·17

Table 2. *The content of some of the ions in unfractionated suspensions and 'cyclophorase' preparations from sheep kidney cortex in the presence of ATP and magnesium at 20°*

All preparations contained 1 mM-Mg^{2+} and 1–2·5 mM-ATP, and were centrifuged at 20°. The concentrations of substrates are those in the final mixture, and concentrations in the particles refer to the centrifuged material.

Expt. no.	Type of preparation and treatment	Apparent pH of Particles	Medium	Content of sodium in (m-moles/kg.) Particles	Medium	Ratio of sodium in: particles / medium	Content of potassium in (m-moles/kg.) Particles	Medium	Ratio of potassium in: particles / medium
1*a*	Twice-washed 'cyclophorase'*	—	—	—	—	—	158	90·5	1·75
b	+Sodium pyruvate (5 mM) +$NaHCO_3$ (12·5 mM); incubated 15 min. before separation	—	—	—	—	—	212	89·0	2·38
2	Unfractionated suspension:								
a	Incubated 15 min. at 20° before separation	7·51	7·53	21·1	18·7	1·12	57·0	61·4	0·93
b	+Sodium α-oxoglutarate (5 mM); separated at once	7·38	7·55	32·2	28·6	1·12	55·5	61·2	0·91
c	+Sodium α-oxoglutarate (5 mM); incubated 15 min. before separation	7·36	7·56	28·1	29·4	0·96	58·2	61·6	0·94

* The sheep kidney had been stored for 24 hr. in a mixture of frozen and liquid 0·9% KCl before use.

Table 3. *The effect of ATP and substrate at 20° on the production of some ionic gradients in 'cyclophorase' preparations from sheep kidney cortex*

The twice-washed 'cyclophorase' used contained initially 13·9 m-moles sodium/kg. in the centrifuged material and 6·9 m-moles sodium/kg. in the medium. The incubated samples all contained 1 mM-Mg^{2+} and 12·5 mM-$NaHCO_3$, and were incubated for 30 min. at 20°. The concentrations of substrates are those in the final mixtures.

Expt. no.	Conditions	Additions (sodium salts)	Content of sodium in (m-moles/kg.) Particles	Medium	Ratio of sodium in: particles / medium	Content of potassium in (m-moles/kg.) Particles	Medium	Ratio of potassium in: particles / medium
1*a*	Aerobic	None	18·2	16·9	1·07	82·6	70·6	1·17
b		Pyruvate (5 mM)	22·1	17·8	1·24	85·5	74·9	1·14
c		ATP (1 mM)	24·7	21·3	1·15	81·5	73·9	1·10
d		Pyruvate (5 mM) +ATP (1 mM)	33·4	23·0	1·43	79·2	74·9	1·05
2*a*	Anaerobic	None	20·4	16·5	1·23	84·5	73·9	1·16
b		Pyruvate (5 mM)	19·5	18·2	1·07	82·3	73·9	1·12
c		ATP (1 mM)	27·8	20·8	1·33	86·7	75·5	1·15
d		Pyruvate (5 mM) +ATP (1 mM)	37·8	23·4	1·61	94·1	74·9	1·26

medium but the gradient decreased on incubation without substrate. In contrast, on incubation in the presence of an oxidizable substrate the 'cyclophorase' could accumulate sodium and potassium against concentration gradients (Tables 2 and 3). Under these conditions the 'cyclophorase' maintained a steady rate of metabolism and of oxidative phosphorylation (cf. Bartley, 1953*b*).

Although these effects were regularly obtained and duplicate samples of the same preparation gave uniform results, samples of apparently similar preparations were rarely comparable quantitatively. Measurements of the sodium, potassium and 'hydrogen ion' contents of the centrifuged material and of the supernatant fluid showed that these ions were often treated differently. As shown in Table 2, Expt. 2, only slight changes occurred in the sodium and potassium concentrations in the particles of an unfractionated suspension, although there were significant changes in 'pH'. (The 'pH' figures given in Tables 2 and 3 are probably not true pH values, but a reflexion of some electrical property of the particles to which the glass electrode is sensitive. But whatever the phenomenon measured by the glass electrode, the apparent pH difference usually varied with the metabolic activity and was always abolished by incubation in the absence of substrate.) Table 3, Expt. 1, shows that accumulation of sodium could take place in the absence of significant changes in the potassium content, whilst Table 3, Expt. 2, gives an example of simultaneous accumulation of sodium and potassium. The addition of substrate could increase a pre-existing gradient (see Table 2, Expt. 1, and Table 3, Expt. 1), but in some cases, e.g. in the anaerobic experiment shown in Table 3, Expt. 2, the sodium gradient was increased by the added ATP rather than by the substrate, though as in Table 3, Expt. 1, the biggest effects were obtained when both ATP and substrate were present.

Concentration of oxidizable organic anions in 'cyclophorase' preparations. The distribution of some oxidizable substrates between 'cyclophorase' preparations and medium is shown in Table 4. The study of the accumulation of oxidizable substrates by the particles is complicated by the rapid removal of the comparatively small amount of intraparticulate substrate by the relatively high activity of the oxidizing enzymes. Thus the intraparticulate concentrations of substrate given in Table 4 must be minimal, and the very low concentration of oxaloacetate may only indicate a very rapid rate of metabolism. Table 4 shows that all the substrates tested, with the exception of oxaloacetate, appear within the particles and in the case of fumarate, when the amount was low, a marked accumulation of the substrate occurred.

The relatively high intraparticulate level of citrate found (about 85% of that in the medium) is of interest in view of Peters's assumption (1952) that citric acid would only slowly penetrate mitochondria.

The gradients given in Tables 1–4 are uncorrected for extraparticulate fluid. The inulin space of a twice-washed, metabolizing 'cyclophorase' preparation was found to be 66%. This high figure suggests that some of the mitochondrial or nuclear volumes are accessible to inulin and perhaps to other solutes of low molecular weight. If this is the case the true gradients would be up to 3 times greater than those recorded. However, it is doubtful whether this figure applies strictly to other sorts of preparation or to non-metabolizing material.

Table 4. *Distribution of oxidizable substrates between 'cyclophorase' particles and suspending medium during oxidation of the substrate at* 20°

A twice-washed 'cyclophorase' preparation from sheep kidney cortex was used; the mixture, which contained 1 mM-Mg^{2+} and 1–2·5 mM-ATP, was incubated usually for a period of about 15 min. before the particles were separated from the medium. The concentrations of substrates are those in the final mixtures and concentrations in the particles refer to the centrifuged material.

Expt. no.	Substrate added	Concentration in: Particles (m-moles/kg.)	Concentration in: Medium (mM)	Ratio: concentration in particles/ concentration in medium	Calculated concentration in particles corrected for loss by oxidation during separation (m-moles/kg.)	Ratio: corrected concentration in particles/ concentration in medium
1	Pyruvate (2·5 mM)* + $NaHCO_3$ (2·5 mM)	0·315	0·305	1·03	0·339	1·11
2	Pyruvate (2·5 mM)	0·297	0·352	0·84	0·365	1·03
3	Fumarate (12·5 mM)	1·46	1·69	0·86	?	?
4	Fumarate (2·5 mM)*	2·08	0·28	7·42	?	?
5	α-Oxoglutarate (12·5 mM)	9·7	9·7	1·00	11·5	1·09
6	Citrate (12·5 mM)	10·8	13·0	0·83	11·2	0·86
7	Oxaloacetate (2·5 mM)	0·239	1·79	0·133	?	?

* The initial substrate concentrations are only approximate.

Table 5. *Effect of substrate on the water content of the particulate fraction of a whole unwashed suspension*

The preparation was fortified with 1 mM-ATP and 1 mM-Mg^{2+} final concentrations; no incubation indicates that the suspension was centrifuged immediately after mixing at 20°.

Substrate added and treatment	Ratio: $\frac{\text{dry wt. of particles}}{\text{wet wt. of particles}} \times 100$
No substrate, no incubation	13·1
No substrate; shaken in O_2 for 15 min. at 20°	11·9
Sodium α-oxoglutarate (5 mM); no incubation	19·4
Sodium α-oxoglutarate (5 mM); shaken in O_2 for 15 min. at 20°	20·2

Water content of kidney particles. The dry matter of 'cyclophorase' preparations centrifuged under different conditions ranged from 8 to 20 %. In most washed preparations addition of substrate had no consistent effect upon the water content, which remained constant within 3 % during the experiment, but when an unfractionated suspension was used, the addition of 0·005 M α-oxoglutarate in the presence of ATP almost doubled the percentage dry weight of the particles and maintained this during an incubation of 15 min. (Table 5). The process of fractionation appears to interfere with mechanisms in kidney particles which reduce the water content in the presence of substrates. These observations may be compared with those of Macfarlane & Spencer (1953), who found a low water content of liver mitochondria when the suspending medium contained AMP and an oxidizable substrate.

Investigations with sheep kidney-cortex mitochondria

Further experiments were carried out with preparations of mitochondria, since it seemed likely that these particles were associated with the ability of 'cyclophorase' preparations to accumulate ions against concentration gradients.

Gradients of cations at 0°. Table 6 gives typical results for the concentrations of sodium, potassium, magnesium and hydrogen ions found in preparations of mitochondria and in the washing fluid immediately after isolation at 0°. The washing fluid was usually sodium or potassium chloride solution (0·9 %, w/v) and no substrates or cofactors were added. The endogenous respiration under these conditions at 0° was 0·5 μl./mg. dry wt./hr. and remained stable for a few hours.

It was found that even after extensive washing with potassium chloride solution, other cations remained 'bound' in the particles. The concentration gradient between particles and the suspending medium was highest at low concentrations of ions, for example for potassium when the particles had been washed with sodium chloride solution, or for sodium when potassium chloride solution had been used for washing. The maximum gradient observed for sodium was 26·5 (sheep kidney mitochondria) and for potassium was 4·75 (rat heart sarcosomes). The sodium gradient was not abolished by freezing the preparation once but disappeared on storing for 10 days at 2°.

Maintenance of gradients and accumulation of cations at 20°. Further experiments were carried out at 20° with mitochondria to which substrates and/or ATP had been added, and which were either centrifuged immediately after mixing or after varying periods of incubation.

Whereas at 20° the initial endogenous oxygen uptake of twice-washed sheep kidney-cortex mitochondria was about 2 μl./mg. dry wt./hr. and became zero after 20 min., the addition of ATP, with magnesium and phosphate, doubled the initial oxygen uptake which again became zero within 20 min. The addition of substrate, as well, produced a steady oxygen uptake of about 10 μl./mg. dry wt./hr. which could be maintained for 2 hr.

As with 'cyclophorase' preparations, it was found that duplicate samples of the same mitochondrial preparation, whether metabolizing or not, gave uniform results, but samples of apparently similar preparations were rarely comparable quantitatively. A typical experiment with mitochondria is given in Table 7, which provides evidence for the active transport during metabolism of sodium and potassium ions against concentration gradients. The untreated material in the presence of ATP, magnesium and phosphate contained more sodium and potassium than the medium. The addition of α-oxoglutarate caused no immediate change in the extra amounts of these ions in the mitochondria, but during incubation for 15 min., either aerobically or anaerobically, the extra sodium and potassium contents were greatly increased. The water content of the centrifuged material remained constant at about 88 % throughout the experiment.

The apparent pH differences between mitochondria and medium were similar to those observed with 'cyclophorase' preparations. They usually varied with the metabolic activity and were always abolished by incubation in the absence of substrate. An apparent pH difference could sometimes be induced in a preparation previously incubated without substrate by the subsequent addition of substrate. Table 8 shows this effect and also illustrates the abolition of the 'pH' difference by freezing and thawing a metabolizing suspension of mitochondria. This is in contrast to the result obtained by freezing and thawing mitochondria which had been washed with 0·9 % (w/v) potassium chloride at 0° (Table 6, Expt. 1).

Table 6. *The content of some of the ions in samples of mitochondria at 0°*

Unless otherwise stated, all preparations were twice washed and were prepared from sheep kidney cortex without prior storage of the kidney. The concentrations in the mitochondria refer to the centrifuged material.

Expt. no.	Type of preparation and treatment	Apparent pH of		Content of sodium in		Ratio of sodium in:	Content of potassium in		Ratio of potassium in:	Content of magnesium in		Ratio of magnesium in:
		Mitochondria	Medium	Mitochondria	Medium	mitochondria / medium	Mitochondria	Medium	mitochondria / medium	Mitochondria	Medium	mitochondria / medium
				(m-moles/kg.)			(m-moles/kg.)			(m-moles/kg.)		
1*a*	Kidney mitochondria untreated	6·87	7·09	15·7	0·59	26·5	102	121	0·84	0·074	0·062	1·19
1*b*	Frozen before separation	6·73	7·00	7·8	0·78	10·0	116·5	113·5	1·02	0·113	0·025	4·50
2	Kidney mitochondria untreated	—	—	—	—	—	134	122	1·09	—	—	—
3	Kidney mitochondria, unwashed and untreated	8·16	8·15	16·2	11·8	1·38	138	103	1·34	—	—	—
4	Kidney mitochondria, washed twice in 0·9% NaCl	—	—	—	—	—	2·59	1·92	1·34	—	—	—
5	Rat heart sarcosomes prepared in saline ethylenediaminetetraacetic acid (Versene)	—	—	66·3	73·5	0·90	149	123	1·21	—	—	—
6	Rat heart sarcosomes prepared in sucrose ethylenediaminetetraacetic acid (Versene)	—	—	29·4	28·3	1·03	1·90	0·40	4·75	—	—	—

Table 7. *Movements of ions in suspensions of sheep kidney-cortex mitochondria during incubation at 20°*

The suspensions of unwashed mitochondria contained 1 mM-Mg^{2+} and 2·5 mM-ATP. The concentrations are those in the final mixture and concentrations in the particles refer to the centrifuged material.

Additions and treatment	Apparent pH of		Content of sodium in		Ratio of sodium in: mitochondria / medium	Content of potassium in		Ratio of potassium in: mitochondria / medium
	Mitochondria	Medium	Mitochondria	Medium		Mitochondria	Medium	
			(m-moles/kg.)			(m-moles/kg.)		
Centrifuged at once	7·39	7·39	20·8	15·5	1·34	88·8	68·7	1·29
Incubated 15 min. 20° before separation	7·51	7·53	—	—	—	—	—	—
Sodium α-oxoglutarate (5 mM); centrifuged at once	7·32	7·48	29·2	24·9	1·17	88·3	71·1	1·24
Sodium α-oxoglutarate (5 mM); incubated 15 min. 20° before separation	7·41	7·49	40·2	26·1	1·54	111	68·5	1·62
Sodium α-oxoglutarate (5 mM); incubated 15 min. 20° anaerobically before separation	7·53	7·65	35·2	25·1	1·41	97·5	71·1	1·37

Table 8. *Changes of* pH *of whole suspensions, of separated mitochondria, and of medium produced by addition of substrate and by freezing*

Twice-washed sheep kidney-cortex mitochondria were used. The 4 ml. of suspension contained 1 mM-ATP, 1 mM-Mg^{2+} and 5 mM phosphate. All pH measurements were made at 23°.

Order of treatments of material	Apparent pH of Suspension	Precipitate	Medium
None	7·48	—	—
Separated	—	7·39	7·39
Remixed and incubated for 15 min. at 23°	7·61	—	—
Separated	—	7·51	7·53
Remixed	7·62	—	—
Sodium α-oxoglutarate added (0·2 ml. of 0·1 M) incubated for 1 min. at 23°	7·62	—	—
Separated	—	7·45	7·61
Remixed	7·60	—	—
Mixture frozen and thawed	7·30	—	—
Separated	—	7·41	7·44
Remixed	7·50	—	—

Table 9. *Changes during incubation of the distribution of* ^{42}K *between mitochondria and medium*

The suspension of twice-washed sheep kidney-cortex mitochondria contained 1 mM-ATP and 1 mM-Mg^{2+}; α-oxoglutarate when added was 12·5 mM, final concentration; the isotope was added last. Concentration in the mitochondria refers to the centrifuged material. The times include the period needed to separate the particles.

Expt. no.	Temp. (°)	Substrate added	Time of incubation (min. sec.)	Concentration of potassium in mitochondria (m-moles/kg.)	Concentration of potassium in medium (m-moles/kg.)	Specific activity (counts/min./mg. potassium) in Mitochondria	Medium	Ratio (specific activity potassium in mitochondria/ specific activity potassium in medium) × 100
1	0	None	1 49	128·5	136·5	11 000	10 700	102
			5 47	156	142	10 400	12 000	87·0
			11 05	133	140·5	12 200	12 300	99·5
			30 53	130·5	137	12 400	12 300	102
2	0	α-Oxoglutarate	1 27	133	137·5	10 500	12 200	86·0
			5 17	111	140·5	13 500	12 200	110·5
			10 35	123	136	12 200	12 600	97·0
			30 31	128	135	11 600	12 900	90·6
3	21	None	1 49	131	135	10 300	10 700	96·4
			5 47	130	136·5	12 600	12 100	104
			11 05	133	137	12 200	13 200	92·5
			30 53	136	141	12 400	12 500	99·3
4	21	α-Oxoglutarate	1 27	129·5	138·5	12 600	12 900	97·6
			10 35	128	137	11 300	12 300	91·5
			30 31	132	139	11 400	10 700	106·5

As shown earlier, twice-washed mitochondria contained more magnesium than the medium, but the differences in concentration between particles and medium were increased on freezing, in contrast to the decreases in the sodium and potassium concentration differences (Table 6, Expt. 1). After the magnesium concentration in the medium had been raised from 0·062 to 1·3 mM, at 20° in the presence of 20 mM potassium phosphate buffer, pH 7·4, and 2·5 mM-ATP, the mitochondria were found to contain 2·7 m-moles magnesium/kg. This figure decreases to 2·1 m-moles/kg. on incubation for 15 min. at 20°, but was maintained at 2·7 m-moles/kg. during incubation in the presence of 5 mM α-oxoglutarate. Thus the large increase in metabolism caused by the substrate (see earlier) did not cause any further increase in the magnesium content of the mitochondria.

Turnover rates of sodium and potassium ions. The values given in Table 6 show that some of the 'intramitochondrial' sodium (Expt. 1) and potassium (Expts. 4 and 6) ions cannot be washed out with a washing fluid containing potassium or sodium ions, respectively. In order to find out whether all the intramitochondrial sodium and potassium is exchangeable in the presence of ATP, magnesium, phosphate and oxidizable substrates the incorporation of radioactive sodium or potassium into the mitochondria after addition to the medium was measured. It so happened in these

experiments that unexpectedly no concentration gradients of sodium or potassium between mitochondria and medium developed. Although the results are rather scattered, it is clear that potassium with or without substrate exchanged rapidly and completely at 0° or at 20° (Table 9). Almost 100% was exchanged by the time of the first measurements (within 2 min.) and this means that the rate was greater than 44 m-moles/kg./min.

The exchange of sodium was affected both by temperature and by the addition of substrate. Without substrate at 0° about 80% of the sodium had exchanged within 40 sec., but the remaining 20% had a far slower exchange rate (Table 10, Expt. 1). At 20° (Table 10, Expt. 4) virtually all the sodium had rapidly and completely exchanged by the time of the first measurement (24 sec.). The addition of fumarate or α-oxoglutarate at 0° (Table 10, Expts. 2 and 3) also rendered the whole of the sodium exchangeable, but the rates at which the exchange occurred were different, being completed by the time of the first measurement (24 sec.) in the case of α-oxoglutarate and not until 2 min. 24 sec. had elapsed in the case of fumarate. In the first minute the rate of sodium exchange increased from approx. 13 m-moles/kg./min. ($Q_{Na}=1600$ μl./mg. dry wt./hr.) to about 29 m-moles/kg./min. ($Q_{Na}=3600$) with fumarate and to at least 39 m-moles/kg./min. ($Q_{Na}=4900$) when α-oxoglutarate was the substrate. These increases were accompanied by an increase in the Q_{O_2} of the preparation of about 0·5 with either substrate at 0°.

Concentration of phosphorus-containing compounds in mitochondria washed four times at 0°. Green, Atchley, Nordmann & Teply (1949) have reported that phosphate remains firmly attached to 'cyclophorase' particles after washing with potassium chloride solution. They thought that the phosphate was chemically bound and called it 'gel-phosphate'. This observation of Green *et al.* (1949) has been confirmed for both 'cyclophorase' and mitochondrial preparations. The concentration of

Table 10. *Changes during incubation of the distribution of* ^{24}Na *between mitochondria and medium*

The suspension of twice-washed sheep kidney-cortex mitochondria contained 1 mM-ATP and 1 mM-Mg^{2+}; substrate when added was 2·5 mM, final concentration. The isotope was added last. Concentration in the mitochondria refers to the centrifuged material. The times include the period needed to separate the particles.

Expt. no.	Temp. (°)	Substrate added	Time of incubation (min. sec.)	Concentration of sodium in mitochondria (m-moles/kg.)	Concentration of sodium in medium (m-moles/kg.)	Specific activity (counts/min./μg. sodium) in Mitochondria	Specific activity (counts/min./μg. sodium) in Medium	Ratio (specific activity sodium in mitochondria/specific activity sodium in medium) × 100
1	0	None	0 24	10·1	10·6	295	408	72·0
			0 39	8·84	10·6	325	413	78·5
			0 54	8·12	10·7	318	401	79·5
			1 24	8·65	9·86	351	432	79·5
			2 24	9·67	9·69	327	428	76·5
2	0	Fumarate	0 24	16·0	16·3	183	242	75·0
			0 39	13·4	16·7	222	249	89·5
			0 54	14·2	16·5	222	252	88·0
			1 24	15·2	16·1	228	254	90·0
			2 24	14·8	16·5	240	241	99·0
3	0	α-Oxoglutarate	0 24	13·0	14·6	265	269	98·8
			0 39	14·1	14·8	266	264	100·5
			0 54	13·2	15·5	328	279	117
			1 24	13·5	14·9	288	270	107
			2 24	13·8	14·6	261	269	97·0
4	20	None	0 24	9·34	10·5	396	362	109
			0 39	9·27	9·43	403	428	95·0
			0 54	9·14	9·87	414	389	106
			1 24	9·03	9·78	389	403	96·0
			2 24	10·2	9·71	374	413	93·0
5	20	Fumarate	0 24	14·6	15·9	252	255	99·0
			0 39	15·8	15·5	243	253	96·0
			0 54	15·9	15·7	236	244	97·0
			1 24	14·8	15·8	248	237	104
			2 24	15·7	16·5	245	227	108
6	20	α-Oxoglutarate	0 24	14·7	14·6	269	267	100·5
			0 39	14·8	14·8	269	268	100
			0 54	15·2	14·8	257	251	102·5
			1 24	14·2	15·0	253	267	95·1
			2 24	14·6	14·4	260	265	101

Table 11. *Intramitochondrial phosphate-containing compounds*

Freshly prepared sheep kidney-cortex mitochondria washed 4 times with 0·9% KCl at 0°. The last washing contained $1·9 \times 10^{-4}$ M phosphate.

Compound	Concentration (m-moles/kg.)
Adenosine triphosphate	0·132
Adenosine diphosphate	0·186
Adenosine monophosphate	0·352
Inosine monophosphate	0·197
Inorganic phosphate	2·51

'gel-phosphate' was found to be dependent on the concentration of phosphate in the medium. It was 1·5–2 times higher in the mitochondria than in the medium at phosphate concentrations between 0·005 and 0·01 M. At the very low concentrations of phosphate ($1·9 \times 10^{-4}$ M) found in the potassium chloride suspending medium the mitochondria gave the values for the phosphate-containing compounds shown in Table 11. In view of the fact that other ions occur at higher concentrations in the mitochondria, it is, perhaps, unnecessary to assume that 'gel-phosphate' is chemically different from inorganic phosphate. The occurrence of inosine monophosphate has not been recorded previously and indicates the presence of an adenylic deaminase. The amount of the inosine monophosphate present is relatively large, being of the same order as the amount of ADP. The proportions of the adenosine compounds found are much nearer the equilibrium for myokinase than those found in the tissues (see Eggleston & Hems, 1952); in whole muscle about 70–80% of the total adenosine phosphates consists of ATP, 20–30% is ADP and there are only traces of AMP (Oliver, 1953). In this connexion the presence of myokinase in the mitochondria (Kielley & Kielley, 1951) is of interest. Compared with whole rat kidney (Oliver, 1953) the values with exception of inosine monophosphate are low, ranging from about one-fifth to one-half. The high concentration of inosine monophosphate probably reflects the relatively high proportion of AMP found.

Accumulation and turnover of phosphate in respiring mitochondria at 20°. Preliminary experiments showed that inorganic phosphate was accumulated by actively metabolizing mitochondria and this accumulation was further studied by incubating a sheep-kidney mitochondrial preparation with a medium containing ^{32}P-labelled orthophosphate and pyruvate. Samples of the reaction mixture were centrifuged at various times to separate particles and medium, and both fractions were analysed for phosphate compounds. In Fig. 1 the phosphate concentrations (curve *A*) and the specific activities (curve *B*) of the two fractions have been plotted as a function of the incubation time. An accumulation of intramitochondrial phosphate occurred within the first 2 min. reaching a maximum at 3·5 min. (Fig. 1*A*). At the same time, the specific activity of the orthophosphate declined (Fig. 1*B*). The fact that the specific activity of the mitochondrial phosphate was lower (62–78%) than

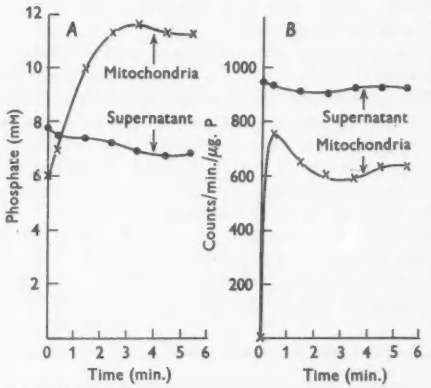

Fig. 1. Changes in the concentration (*A*) and specifi activity (*B*) of the inorganic phosphate in twice-washed sheep kidney-cortex mitochondria and in the medium during aerobic incubation in the presence of radioactive phosphate. The medium contained 2·5 mM sodium ATP; 5 mM potassium phosphate buffer, pH 7·4 (including $^{32}PO_4$); 60 mM-KCl; 1 mM-$MgCl_2$; 8 mM-$NaHCO_3$; 5 mM sodium pyruvate; gas, air; temp., 20°.

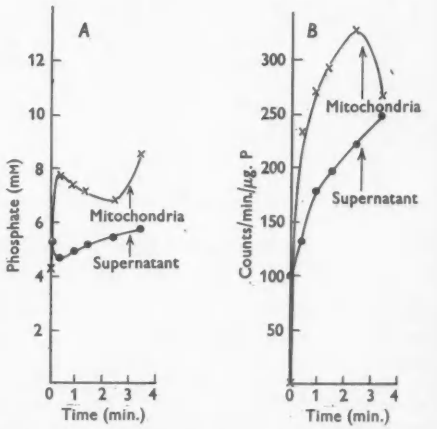

Fig. 2. Changes in the concentration (*A*) and specific activity (*B*) of the inorganic phosphate in twice-washed sheep kidney-cortex mitochondria and in the medium during aerobic incubation in the presence of radioactive adenosine triphosphate. The experimental conditions were similar to those in Fig. 1 but $^{32}PO_4$ was replaced by [$^{\beta\text{-}\gamma}$.$^{32}P_2$] adenosine triphosphate.

that of the phosphate in the medium and fell despite a net increase of phosphate, and further the constancy of specific activity outside indicates that most of the phosphate accumulating within the mitochondria did not come from the external orthophosphate directly. The only possible source of this non-radioactive phosphate was the added ATP, which must have penetrated as such through the surface of the mitochondria and released phosphate within it, thus acting as a phosphate-carrier. It should be realized that the calculated specific activity of the mitochondrial phosphate includes the activity due to the extramitochondrial phosphate located in the spaces between the mitochondria.

To confirm these conclusions the experiment was repeated with ^{32}P-labelled ATP. In this case the mitochondrial phosphate became radioactive almost at once and the specific activity became higher than that in the medium; as before, the absolute amount of mitochondrial phosphate increased (Fig. 2). This confirms that mitochondrial phosphate is derived from external ATP. The adenosine compounds inside the mitochondria isolated at the end of the incubation consisted of ADP and AMP in approximately equimolecular proportions (1·5 mM); no ATP was found. As the ATP in the incubating medium remained as ATP and showed no breakdown to ADP or AMP, but at the same time reached isotopic equilibrium, it is very probable that the dephosphorylation of ATP and rephosphorylation of ADP both occur at the membrane separating phosphate inside the mitochondria from the external medium.

DISCUSSION

Turnover rates of sodium and potassium. The distribution of radioactive potassium shows that within the experimental error all the potassium of kidney-cortex mitochondria was readily exchangeable, but it was not possible to investigate the effects of metabolism on the rate of exchange because this rate was already immeasurably high in the absence of substrate at 0°. In view of the short path of diffusion (1–2 μ.) the attainment of isotopic equilibrium within 2 min. is not surprising. An unexpected finding was that in the absence of substrate about 20 % of the sodium did not exchange rapidly at 0°, and that the rate of turnover of sodium was increased on addition of oxidizable substrates. It is unlikely that the increase in the oxygen uptake caused by the substrate is directly related to the higher rates of sodium turnover, because the two substrates used produced similar increases in oxygen uptake but different increases in the rate of sodium turnover, which were very high in relation to the increases in oxygen uptake.

Since the phosphorylation quotient is higher during oxidative decarboxylation of α-oxoglutarate than during oxidation of fumarate (Ochoa & Stern, 1952) this might explain the greater effectiveness of α-oxoglutarate in increasing the turnover rate of sodium ions. However, it is unlikely that the effects are caused solely by the energy available from the ATP produced, because even with a phosphorylation quotient of 4 for α-oxoglutarate only about 15 cal. would be available to cause the to-and-fro exchange of 1 mole of sodium (cf. Burton & Krebs, 1953). This could only concentrate ions by a factor of 1·03 even if used with 100 % efficiency.

It therefore seems likely that the steady-state exchange under these conditions is not the result of an active accumulation and a passive leakage, and that the to-and-fro movements must be coupled energetically in a similar way to the movements of an ion between a salt solution and an ion exchange resin in equilibrium with it (Boyd, Schubert & Adamson, 1947). The substrate or one of its metabolic products may act on the mitochondria and result in an increased permeability, possibly by the production of carrier molecules which can maintain an 'exchange diffusion' of the type postulated by Ussing (1949). If this be true, changes in the ratio of Q_{Na}/Q_{O_2} become of only indirect interest. Further work is needed on the changes in the rate of sodium exchange aerobically and anaerobically with a variety of substrates to throw light on this phenomenon.

Concentration gradients of inorganic ions. The concentration gradients recorded in this paper for kidney mitochondria are similar to those recently found for liver mitochondria by Macfarlane & Spencer (1953) and Spector (1953). The very high ratio of internal to external sodium and the fairly high absolute concentration (15·7 mM) that could remain after three washings with 0·9 % KCl at 0° indicate that this sodium is 'bound' in this particular set of circumstances and cannot exchange with external potassium ions. This excess amount of sodium in the mitochondria relative to the medium was not observed at higher sodium concentrations or in the presence of ATP, magnesium and phosphate (Table 6, Expt. 3; Table 7). Similarly, potassium remaining in the mitochondria washed with 0·9 % NaCl cannot be replaced by sodium. However, in preparations which were metabolizing in the presence of substrate there was no evidence for sodium or potassium which could not exchange for external sodium or potassium, respectively. The firm way in which sodium and potassium are attached to the mitochondria in the absence of substrate is paralleled by the firm attachment of phosphate under similar circumstances. This firmly attached phosphate may be partly explained by the observations made in this paper

that phosphate as such does not easily enter the mitochondria and that ATP acts as a carrier of this ion.

While these small quantities of sodium or potassium can be retained by the particles without expenditure of energy, the accumulation of sodium and potassium at higher external concentrations (20–90 mM) and the maintenance of concentration gradients at these levels are processes requiring energy. In contrast, although magnesium was always found in higher concentration in the mitochondria, there were no clear effects of metabolism on the concentration gradient or the absolute content of this ion. It is possible that the gradient is maintained by a type of 'binding' process similar to that described for calcium by Slater & Cleland (1953).

Active transport of material is here taken to mean the development of concentration gradients by processes ultimately dependent upon metabolism, and this includes movements along an electrochemical gradient if the electric potential itself is maintained by metabolism. The demonstration of the mere existence of a concentration gradient in a mitochondrial or 'cyclophorase' preparation (see Tables 1 and 6) does not therefore necessarily mean that active transport has occurred, because electric charges independent of metabolism may have 'bound' the ions to the particles. The crucial test is the effect of metabolism on the distribution of ions in any given tissue preparation.

Thus, active transport of what is measurable as hydrogen ions is demonstrated in Tables 7 and 8 (mitochondria); of sodium in Table 3, Expts. 1 and 2 ('cyclophorase') and in Table 7 (mitochondria); of potassium in Table 2, Expt. 1, and Table 3, Expt. 2 ('cyclophorase') and in Table 7 (mitochondria). The accumulation during metabolism of phosphate is shown in Figs. 1 and 2 (mitochondria) and of pyruvate, fumarate and α-oxoglutarate in Table 4, ('cyclophorase').

In all cases the results have purposely been given in the most disadvantageous way for demonstrating gradients—m-moles/kg. centrifuged material—and these gradients must be much higher for the particles themselves or for the water in the particles.

The observation of a simultaneous accumulation of cations and anions excludes a *simple* Donnan effect as the explanation of the maintenance of the gradients. There remains the possibility that the mitochondria behave like a more complex Donnan system with two immobile electrolytes of opposite charge such as mixtures of cation and anion exchange resins which can maintain high gradients of both cations and anions in salt solutions (cf. Biodeminrolit; the Permutit Co. Ltd., London). In this case metabolic energy might be needed to generate or maintain the immobile charged centres. The actual steady-state exchanges could then take place without further energy supplies.

Inulin space. The large inulin space of a metabolizing centrifuged 'cyclophorase' preparation (over 60 %) makes it possible that some of the mitochondrial volume is accessible to small molecules. Thus any membrane which limits diffusion may lie well within the visible structure, or, more probably, the mitochondria have a sponge-like structure with a large 'surface' area. This concept is supported by recent electron micrographs by Sjöstrand & Rhodin (1953) which show that kidney mitochondria are divided into a series of compartments; some of these may be more easily accessible to small molecules than others.

Phosphate transport. The observation that ATP is the carrier for the transport of orthophosphate across the mitochondrial membrane is in agreement with the observations of Gourley (1952), who showed that the same phenomenon occurs in red cells. Recently, Crane & Lipmann (1953) also found that there was a delay in the equilibration of intramitochondrial orthophosphate when ^{32}P-labelled orthophosphate was added to the incubation medium.

Accumulation of oxidizable substrates. The concentrations of the substrates found in the centrifuged particles must be lower than the concentration in the respiring material owing to loss by oxidation during the time required to separate the particles and to stop metabolism. An allowance for the substrate oxidized has therefore been made on the basis of the times taken, the rates of oxygen uptake, and the following assumptions: (*a*) when pyruvate is the substrate the ratio of oxygen consumed/pyruvate removed will depend on the bicarbonate concentration (Bartley, 1953*a*); (*b*) the oxidation of α-oxoglutarate is largely a one-step conversion into succinate (Bartley, 1954); (*c*) citrate is oxidized completely to carbon dioxide and water (Krebs *et al.* 1953). The corrected concentrations are shown in Table 4, except for fumarate and oxaloacetate for which insufficient information is at present available to make a correction for the amount lost.

Since there was 66 % extraparticulate water in the centrifuged material these calculations show that fumarate, pyruvate and α-oxoglutarate can be concentrated inside the particles, whilst citrate occurs at lower concentration than in the medium, and oxaloacetate apparently does not appear in the particles.

SUMMARY

1. Techniques are described for the rapid isolation from the medium of actively metabolizing sheep-kidney mitochondria and 'cyclophorase' preparations (largely mitochondria and nuclei). The particles were spun down in 24 sec. and the medium was removed in about 2 min.

2. The mitochondria could maintain concentration gradients of sodium, potassium, magnesium and phosphate under a variety of conditions. The maximum ratios of internal concentration/external concentration were obtained at 0° in the absence of substrate or cofactors when the concentrations in the external medium were very low. The excess contents of these ions in the particles were thus small.

3. At higher concentrations in the presence of ATP, magnesium, phosphate and oxidizable substrate the initial ratios in the actively metabolizing preparations at 20° were always low, but, in the case of sodium, potassium and phosphate, were increased during incubation. Under these conditions the excess contents of these three ions were higher than at 0° or in the absence of metabolism at 20°.

4. Apparent pH differences of up to 0·4 unit between the mitochondria and the suspending medium were found with a glass electrode. These differences could be developed during metabolism and abolished by incubation without substrate.

5. The turnover rates of the sodium and potassium in the mitochondria were very fast. The rate of exchange of potassium was always greater than 44 m-moles/kg./min. and was too fast to be measured. The exchange rate of sodium in the first minute at 0° in the absence of added substrate was approx. 13 m-moles/kg./min.; the addition of 0·0025M fumarate increased this rate to about 29 m-moles/kg./min.; 0·0025M α-oxoglutarate increased it to a rate, greater than 39 m-moles/kg./min., which was too fast to be measured and which also occurred in the presence or absence of substrate at 20°. At 0° without substrate about 20% of the sodium had a very slow rate of exchange.

6. Isotope data indicate that inorganic phosphate accumulating in the mitochondria on incubation in the presence of ATP and substrate is derived from ATP. ATP is thus a phosphate carrier.

7. Freshly prepared mitochondria contained 0·132 m-mole ATP/kg., 0·186 m-mole ADP/kg., 0·352 m-mole AMP/kg., 0·197 m-mole IMP/kg., and 2·51 m-moles orthophosphate/kg.

8. α-Oxoglutarate, fumarate and pyruvate were concentrated during metabolism by 'cyclophorase' preparations. Citrate was found in lower concentration and oxaloacetate was apparently excluded.

9. The dry weight of the particles prepared under different conditions varied from 8 to 20%. In an unfractionated suspension the dry weight was increased by 54% in the presence of substrate at 20°.

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A Rotographic Study of Mutarotase

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Glucose oxidase is a specific enzyme which catalyses the oxidation (dehydrogenation) of glucose. It was studied in detail during the past fifty years and was proved to be identical with penicillin A, penicillin B and Notatin (see Theorell, 1951). It has been prepared in a highly purified form by Coulthard *et al.* (1945), and its characteristics have been firmly established by Keilin & Hartree (1948*a*). Its specificity is truly remarkable, as shown by these authors (Keilin & Hartree, 1952*a*), in that essentially only β-D-glucose is oxidized by the enzyme. In the course of these studies, they noted certain irregularities and they concluded that some glucose oxidase preparations contained another agent, which they called mutarotase. This was in accordance with an earlier observation by Bentley & Neuberger (1949). Keilin & Hartree (1952*b*) indicated, in their succeeding paper, that this agent is a different entity from glucose oxidase and it is probably an enzyme which catalyses the mutarotation of glucose. Based on the molecular weight determination of Cecil & Ogston (1948), they estimate that mutarotase has the remarkably high catalytic coefficient of 2×10^5 min.$^{-1}$, as compared to the best inorganic catalyst, namely the hydroxyl ion, with a coefficient of $8{\cdot}8 \times 10^3$ min.$^{-1}$. For this reason it seemed desirable to study this enzyme in more detail.

An additional impetus to this work was our concern with automatic-recording polarimetry, which is a method of choice in such investigations. Automatic-recording polarimetry—'rotography', in short—is based on our instrument which we have described previously (Levy, Schwed & Fergus, 1950). By its use difficult kinetic studies can be performed with ease and this has been demonstrated with the penicillin—penicillinase system (Levy, 1951). However, owing to the nature of that problem, reactions of essentially only zero order were considered (Levy, 1950), and therefore the problem of mutarotase seemed a suitable test for the application of rotography to first-order kinetics.

EXPERIMENTAL

General technique

The automatic-recording polarimeter, or rotograph, was used in the present work as described previously (Levy *et al.* 1950). It was necessary, however, to construct an anaerobic handling system because the mutarotase used is present as an impurity of glucose oxidase and the activity of the latter had to be eliminated by the exclusion of oxygen. This was accomplished by the use of a polarimeter flow cell of 4 dm. length equipped with plane-parallel windows. This cell was constructed and generously supplied by O. C. Rudolph & Sons, Caldwell, New Jersey, U.S.A. To the inlet of this tube was connected a sintered glass filter and separating funnel,

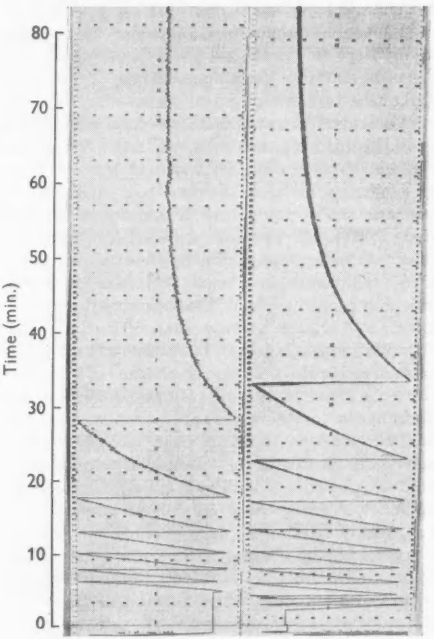

Fig. 1. The mutarotation of α-D-xylose. The left-hand curve shows the changes in optical rotation of 0·1 M xylose solution containing 0·06% glucose oxidase in 0·005 M phthalate buffer, pH 4·7 at 25°. The right-hand curve shows the changes occurring in the absence of enzyme. During the initial period of 3–5 min. the curves are irregular owing to the time required for mixing the solutions and the attainment of balance of the instrument. Each subsequent cycle represents 0·5 angular degree. The results plotted in Fig. 2 represent the changes in optical rotation, expressed in scale divisions, taking place between times subsequent to the attainment of balance and t_∞.

and water-saturated N_2 was fed under pressure to the top of the funnel and the outlet of the tube through stopcocks. The substrate was first placed into the funnel and, by letting N_2 into the outlet of the tube, the system and the solution were cleared of air. After about 15 min. the enzyme preparation was added. The gas flow was maintained and so rapid mixing occurred. By reversing the gas flow the sample was filtered through the sintered glass filter and pumped under N_2 into the polarimeter tube. A rotogram was then prepared. The appearance of such a record is shown in Fig. 1, which represents the mutarotation of xylose with and without mutarotase. By plotting the corresponding values of the abscissa versus time on a semi-logarithmic paper the first-order rate constant can be evaluated graphically or according to the equation:

$$k = \frac{2{\cdot}303}{t_2 - t_1} \log_{10} \frac{\text{divisions } (t_\infty) - \text{divisions } (t_2)}{\text{divisions } (t_\infty) - \text{divisions } (t_1)}.$$

Such a plot, using the data of Fig. 1, is shown in Fig. 2 with the points used for its construction indicated. These points are chosen arbitrarily and are only shown to indicate the precision usually obtained. If a large scatter is encountered, any number of additional points can be used and thus fuller advantage taken of the continuous record. Using this general procedure a large number of mutarotation experiments were performed. To assess the catalytic activity, the rate constant of mutarotation of a control (k_c) and that of the accelerated reaction (k_a) was used to express the acceleration (A), where $A = \dfrac{k_a - k_c}{k_c}$.

The reproducibility of the rate constant depends on various factors. Usually the instrumental precision is not limiting. However, in very slow reactions the long-range stability is of importance. Conversely, in very rapid reactions the evaluation of the record is difficult. Turbid or coloured solutions may limit the signal and precision, whereas temperature fluctuation alters the rate without affecting the precision. Because of this complex situation an arbitrary decision to consider accelerations of 0·05 as the limit of reproducibility was made. When comparison is only between two runs, differences are not considered significant unless the acceleration exceeds 0·1. This limit is rather conservative, but it was found not to be confining, as will be evident.

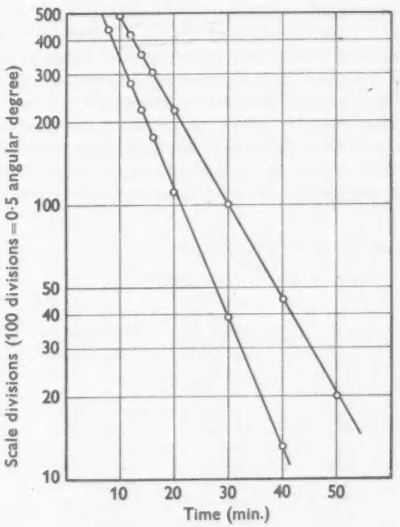

Fig. 2. The mutarotation of xylose. Upper curve without, lower curve with mutarotase (glucose oxidase). Data plotted from Fig. 1.

Materials

Enzymes. Several lots of commercial glucose oxidase were tested for mutarotase activity but no activity was found by the rotographic technique. These were samples of 'Deoxygenase' produced by Takamine Laboratory, Inc., Clifton, New Jersey, U.S.A., and supplied through the courtesy of Dr E. G. Snyder. This finding was further verified qualitatively by measuring the oxidative power of these preparations using equilibrium glucose and α-glucose. Since much slower rates resulted with the latter, the presence of a sizeable quantity of mutarotase was excluded. It was concluded that mutarotase was either lacking due to the manufacturing process or because *Aspergillus niger*, from which the oxidase had been prepared, does not produce mutarotase. The work of Keilin & Hartree (1952*a*, *b*) was done with a highly purified product obtained from *Penicillium notatum* fermentation by Boots Pure Drug Co. Ltd. Such a preparation was generously supplied to us by Dr W. F. Short and the work reported here was carried out with this specimen. It was marked 274–56, 5/9/42, and with glucose as a substrate it exhibited $Q_{O_2} = 50000$ µl./mg./hr. The preparation 233–237, used by Keilin & Hartree, under essentially identical conditions was reported to show $Q_{O_2} = 40000$ µl./mg./hr. The mutarotase activities were also very similar, and it was concluded that these two lots are essentially identical.

The α-amylase used in one experiment was a highly purified preparation of mould amylase which is marketed for analytical purposes by General Biochemicals, Inc. The pepsin, used in another experiment, was of the same manufacture and was a twice-crystallized preparation.

Sugars. These were analytical grade chemicals. α-D-Glucose was prepared from such grade D-glucose (Mallinckrodt Chemical Works, New York, U.S.A.) in accordance with standard procedure (Hudson & Dale, 1917). It was dried *in vacuo* to constant weight.

D-Fructose (Eastman no. 679) was dried *in vacuo*. It consisted almost entirely of the β-form.

α-D-Mannose was obtained from Nutritional Biochemicals Corp. Because of the significance of the results obtained with this sugar, as shown below, these experiments were repeated with another preparation from Difco Laboratories.

β-Lactose was prepared from an Eastman preparation by recrystallization and drying *in vacuo*. The resulting preparation was tested for glucose content by a method essentially as developed by Keilin & Hartree (1948*b*). It showed a glucose content of less than 0·1%.

β-Maltose (Eastman no. 154) was also tested by this procedure. Its glucose content was found to be less than 0·5% and it was used without purification.

α-D-Galactose was tested in a like manner and was found to contain 3·7% of glucose. The high glucose content of a

preparation labelled 'Free from glucose' (Pfahnstiehl Chemical Co.) is not surprising, since Keilin & Hartree (1948*a*) reported 4% glucose in 'pure' commercial galactose. This preparation could be used, nevertheless, because glucose and galactose were found by us to exhibit similar mutarotation, and the small amount of glucose did not cause any detectable error.

α-D-Xylose (Matheson Co. no. 2717) contained no detectable amount of glucose.

All other chemicals were analytical grades and merit no discussion.

RESULTS

Effect of mutarotase on glucose

Keilin & Hartree (1952*b*) have reported in their basic work on mutarotase that at pH 5·6 in 0·2 M phosphate buffer at 20° the rate constant of the mutarotation of α-glucose is 0·0658 min.$^{-1}$. They also determined the acceleration of this mutarotation at various concentrations of their mutarotase preparation. At 0·06% the value was about 0·139 min.$^{-1}$ (by interpolation of their results) representing an acceleration of about 1·1. Since the rotograph was operated at a higher temperature (about 24° in these preliminary experiments) the effect of temperature needs consideration. In general, mutarotation of carbohydrates shows a temperature coefficient for 10° of about 2·5, and Isbell & Pigman (1937) give the figure of 2·6 for α-D-glucose around room temperature. Using this figure, the data of Keilin & Hartree (1952*b*) correspond to about 0·11 min.$^{-1}$ for α-glucose and 0·23 min.$^{-1}$ for the accelerated run at this higher temperature. Using the same buffer, the rate constant for glucose was found by us to be 0·11 min.$^{-1}$ and for the mutarotase-accelerated reaction, 0·22 min.$^{-1}$ (acceleration, 1·0). Thus, not only were their findings verified but it was evident that the two preparations are virtually identical with respect to mutarotase activity (cf. Experimental section).

Effect of metal ions

Before proceeding further with the studies of mutarotase, certain aspects of the mutarotation of glucose were considered. Isbell & Pigman (1937) state that 'the effect of traces of heavy metals on the rates of mutarotation is pointed out and attention is directed to errors caused by the use of nickel-plated brass tubes in mutarotation measurements'. It seemed important to verify this statement. In these experiments 0·005 M phthalate buffer of pH 4·6 was used as was done by Isbell & Pigman. The mutarotation constant for α-glucose at 20° is given by them as 0·0146 min.$^{-1}$, which can be estimated to correspond to about 0·026 min.$^{-1}$ at 25°. This is within experimental error of our values, shown in Table 1. The salts of heavy metals used were cupric acetate, zinc acetate, nickel chloride and mercuric chloride. Also listed is an experiment with ethylenediaminetetraacetic acid (Versene). Gluconic acid, as its sodium salt, was also included in this set of experiments because of its possible presence if, owing to faulty operation, some oxidation of glucose occurs.

Inspection of the first section of the table shows excellent reproducibility, which may, however, be fortuitous. The second section shows that the four heavy metals, even in relatively high trace concentrations (0·001 M) do not alter the mutarotation rate.

It must be appreciated that the addition of a sizeable amount of any ion or salt will cause significant acceleration, e.g. from the data cited in Tables 1 and 3, it can be calculated that the rate of mutarotation of glucose in 0·2 M phosphate buffer is more than 4 times that in 0·005 M phthalate buffers. The nature of the salt or ion (as it affects the dissociation constant) is of importance, as was pointed out by many workers, but within the limit of significance as defined, and in the practical ranges as used, such anions as phosphate, phthalate and gluconate show

Table 1. *The effect of salts on the mutarotation of* 0·1 M *glucose at* 25°

Molarity of phthalate buffer ($\times 10^{-3}$)	Addition*	pH	k (min.$^{-1}$)	Acceleration
5	—	4·6	0·0247 0·0245	— —
7	—	4·7	0·0246	—
5	$Zn(C_2H_3O_2)_2$	4·7	0·024	−0·04
5	$Cu(C_2H_3O_2)_2$	4·7	0·023	−0·09
5	$NiCl_2$	4·5	0·025	0
5	$HgCl_2$	4·4	0·027	+0·08
5	Ethylenediaminetetraacetic acid	4·8	0·026	+0·04
5	Sodium gluconate†	3·7	0·0235	−0·06
2·5	Sodium gluconate‡	3·6	0·025	0

* All 0·001 M except gluconate (see below).
† Anomalous result owing to mutarotation of the gluconate (0·002 M).
‡ Equilibrium gluconate (0·0025 M).

negligible variations. However, if the gluconate itself is undergoing mutarotation anomalous rotograms may be obtained (see Table 1).

pH *optimum of mutarotase*

With this background information, the problem of mutarotase was attacked. A point of importance was the pH optimum of this agent. Keilin & Hartree (1952*b*) used pH 5·6 in their work because the optimum activity of glucose oxidase is at that point. Isbell & Pigman (1938) recommended pH 4·2 or 4·6 for mutarotation studies in general, because of the minimal rates in that range. For the same reason they employed extremely dilute phthalate buffers. It was realized that a generally valid pH optimum for mutarotase activity would not be established while working with a preparation of glucose oxidase which contains but a small portion of mutarotase. However, for the purpose of this study it was deemed necessary to obtain a practical approximation, at least in the neutral-to-acid range, where the lowest natural mutarotation rates occur.

In these experiments a stock solution of glucose oxidase was used in 0·01 M phthalate buffer of varying pH. The solutions were 0·1 M with respect to glucose. The results are shown in Table 2. The agreement with the published pH dependence of the mutarotation of glucose (Isbell & Pigman, 1938) is good. It is also indicated that the pH optimum of mutarotase may be somewhat higher than that of glucose oxidase and possibly somewhat flatter, although in view of the impurity of the preparation (with respect to mutarotase) these conclusions must be considered tentative. It was concluded, however, that further experiments should be carried out at the plateau of maximum activity where the pH dependence is at a minimum. The advantage of using dilute buffer to magnify the accelerating effect of mutarotase is also clear.

Table 2. *The effect of* pH *on the activity of mutarotase*

0·1 M Glucose in 0·01 M phthalate buffer at 25°, glucose oxidase, 0·0294 %.

	k (min.$^{-1}$)		
pH	Glucose	Glucose + enzyme	Acceleration
6·0	0·0262	—	—
5·8	—	0·0937	2·60*
4·7	0·0245	0·0833	2·40
4·0	0·0245	0·0741	2·02
3·2	0·0258	0·0495	0·92

* Obtained by graphical interpolation of the results for the uncatalysed mutarotation.

Nature of mutarotase

The chemical nature of mutarotase may also merit some discussion. Keilin & Hartree (1952*b*) do not make any statement, but their choice of name implies a suggestion of its enzymic nature. Moreover, the tremendous activity (on a molar basis) almost precludes any known inorganic catalyst. They also showed that mutarotase is heat labile (although not to the same extent as glucose oxidase) and is not diffusible. This proof was extended in the present work. To 2 ml. of the glucose oxidase stock solution (0·0294 %)* and 50 ml. of buffer, pH 3·2, crystalline pepsin (15 mg.) was added and the mixture digested at 25° for 45 min. Subsequently, a rotogram with 0·1 M glucose was prepared. The rate constant of mutarotation was found to be 0·0301 min.$^{-1}$. Since the catalysed pH 3·2 run (Table 2) represents a control, it was calculated that owing to this treatment over 81 % of the mutarotase activity was lost. This is further proof of the protein nature of mutarotase.

In this connexion a recent paper by Blom & Schwarz (1952) is of interest. In discussing the actions of α- and β-amylases on starch, they propose 1:5-glucofuranosidic linkages in starch, together with the accepted 1:4- and 1:6-linkages. In discussing this hypothesis and the possible degradation mechanisms, Hopkins (1953) postulates 'mutarotase' activity for α-amylase but doubt about this activity has been expressed in a later (personal) communication from Prof. Hopkins. Considering the fungal source of mutarotase, it would not seem impossible that actually α-amylase is involved, although it is unlikely that this would have escaped the attention of previous investigators. To test this possibility the following experiment was carried out. To 50 ml. of 0·1 M α-D-glucose in 0·01 M phthalate buffer, pH 6, was added 1 ml. of a solution containing 30·1 mg. of amylase. The rate constant was found to be 0·0267 min.$^{-1}$, which is well within analytical error of the control (see Table 2) of 0·0262 min.$^{-1}$. In view of the fact that half the weight of glucose oxidase, which contains but a small portion of mutarotase, showed an acceleration of 2·6 under similar conditions (which is more than 100 times that caused by amylase if the figure 0·0267 is taken at face value), one cannot consider α-amylase as 'mutarotase'. This does not mean that α-amylase preparations may not accelerate mutarotations, as do practically all ionized compounds, nor is any criticism of this new theory of starch structure implied. It seems, however, that mutarotase is a unique entity and most probably an enzyme which accelerates the mutarotation of glucose to an extent not encountered heretofore.

* Final concentration.

Specificity of mutarotase

As a proof that glucose oxidase and mutarotase are different entities, Keilin & Hartree (1952*b*) showed that a number of equilibrium sugars inhibit mutarotase action while scarcely affecting that of glucose oxidase. This pointed to the possibility that the sugars may be substrates of mutarotase. To explore this, lactose, maltose and xylose were tested under conditions similar to those employed by Keilin & Hartree, as shown in Table 3.

These results indicated that there is indeed an accelerating action on the mutarotation of sugars other than glucose. However, the effect was close to the limit of discrimination by the technique. Therefore more favourable conditions were chosen for subsequent experiments, as discussed above. The concentration and pH of the buffer were decreased and 0·005 M phthalate buffer of pH 4·7 was used throughout. The concentration of sugars (0·1 M) was maintained and also the concentration of glucose oxidase (0·06 %). The selection of the sugars was planned in such a fashion as to give most information on specificity. The individual runs were evaluated as shown above (cf. Figs. 1 and 2) and the results are summarized in Table 4.

Table 3. *The action of mutarotase on various aldoses*

0·1 M Sugar solutions in 0·2 M phosphate buffer, pH 5·6, with 0·06 % glucose oxidase.

	k (min.$^{-1}$)		
Substrate	Enzyme absent	Enzyme present	Acceleration
α-D-Glucose	0·11	0·22	1·0
β-Lactose	0·084	0·11	0·31
β-Maltose	0·0098	0·011	0·12
α-D-Xylose	0·37	0·42	0·14

Table 4. *The action of mutarotase on sugars*

0·1 M Sugar solutions in 0·005 M phthalate buffer, pH 4·7, with 0·06 % glucose oxidase.

	k (min.$^{-1}$)		
Sugar	Enzyme absent	Enzyme present	Acceleration
α-D-Glucose	0·025	0·13	4·20
β-D-Fructose	0·17	0·18	0·05
α-D-Xylose	0·079	0·11	0·39
α-D-Mannose	0·064 0·066	0·064 0·066	0
Glucosamine	0·028	0·028	0
α-D-Galactose	0·028	0·166	3·37
β-Maltose	0·021	0·027	0·29
β-Lactose	0·018	0·042	1·33

DISCUSSION

Mutarotation is a rather general term not confined to the chemistry of sugars and which indicates merely that there is a spontaneous change in optical activity. Even in the case of the carbohydrates there are several different reactions which can cause mutarotation. In this branch the most common cause of mutarotation is the change between α- and β-configuration of sugars. However, mutarotation does also occur with changes in ring structure, i.e. pyranose and furanose structures. It seemed unlikely that mutarotase should catalyse such diverse phenomena and the results with fructose indicate that the pyranose–furanose transformation is not accelerated. Conversely, the acceleration of the $\alpha \rightleftharpoons \beta$ type mutarotation appears to be quite general. Obviously α-D-glucose is the preferred substrate. However, α-D-galactose is almost equally acceptable (Table 4), presumably due to the close structural similarity. Apparently the reverse symmetry on the fourth carbon atom is of small importance. It is noted, however, that the linking of a second sugar at that point reduces the effectiveness of mutarotase and, surprisingly, in this case the symmetry appears to have a pronounced effect (cf. lactose and maltose). A large depression of activity is also caused by lack of the sixth carbon, as shown with xylose which is otherwise identical in configuration with glucose. However, all of these aldoses are acted upon by mutarotase to a large extent. The deciding locus of action appears to be the second carbon atom. Reverse symmetry to glucose at that atom abolishes mutarotase action completely (mannose) and the substitution of the hydroxyl group, at that point, by an amino group equally nullifies action (glucosamine).

The outlines of specificity are clear and other sugars are expected to fall into this pattern. The relative lack of specificity of mutarotase is distinct from that of glucose oxidase and this leaves no doubt that they are two distinct enzymes. This lack of specificity seems to justify also the term 'mutarotase', which would seem too broad if only glucose were acted upon.

It was noted that in order to maintain comparable conditions in these studies of specificity, the concentrations of the enzyme and the sugars were kept constant, which resulted in a tenfold range of reaction rates (0·018 min.$^{-1}$ to 0·18 min.$^{-1}$). The fastest changes were thus of the order of 0·25 angular degree/min.$^{-1}$ which could not have been followed visually with any degree of precision. The slowest changes necessitated many hours of continuous recording, which would have represented a formidable task by conventional visual polarimetry. Therefore it appears that automatic operation, or rotography, is of value in this type of

work and it is recommended for all applications where continuous changes of optical activity are involved.

SUMMARY

1. No evidence was found that heavy metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+} and Hg^{2+}) catalyse the mutarotation of glucose.

2. The strong catalytic action of mutarotase was verified. That pepsin largely destroys this activity may be taken as additional proof of the protein nature of mutarotase.

3. α-Amylase was found to have no mutarotase activity.

4. The specificity of mutarotase was investigated in greater detail. It appears that the enzyme catalyses only some $\alpha \rightleftharpoons \beta$ transformations. Of the aldoses tested, glucose and galactose seem to be the best substrates, indicating that the configuration at the fourth carbon atom is not decisive. However, the mutarotation of the disaccharides lactose and maltose is accelerated to a lesser degree. The lack of a hydroxymethyl group on the fifth carbon atom (xylose) also reduces the activity. The configuration at the second carbon atom of the sugar molecule is decisive, since neither mannose nor glucosamine serves as a substrate. These studies indicate the range of specificity of mutarotase.

5. An incidental outcome of this work was that it demonstrated the general utility of automatic-recording polarimetry or rotography.

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The Effect of Certain Barbiturates on the Porphyrin Metabolism of Rabbits

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Since the introduction of the barbiturates to clinical medicine in 1903, there have been conflicting reports on their possible relation to human acute porphyria. Dobrschansky (1906) described a typical case of acute porphyria, without paralysis, occurring in a patient after prolonged administration of diethylbarbituric acid. Haxthausen (1927) reported the development of skin photosensitivity and the presence of excessive amounts of porphyrins in the urine of an epileptic who had been taking ethylphenylbarbiturate for some time. Eliaser & Kondo (1942), Denny-Brown & Sciarra (1945), Prunty (1946) and Jørgensen & With (1947) have described deterioration in the clinical state of acute porphyria patients with the development of severe or even fatal paralysis, after the administration of certain barbiturates, among which were ethylphenylbarbituric acid and ethyl(1-methylbutyl)barbituric acid. Waldenström (1939, 1940) was convinced that barbiturates may precipitate attacks in cases of latent porphyria and that they seriously affect the prognosis of the disease. On the other hand, Günther (1922), Turner (1938) and Discombe & D'Silva (1945) failed to note any relation between barbiturates and acute porphyria.

Information on the relation of barbiturates to normal human porphyrin metabolism has been obtained from observations on the urinary porphyrin excretion in cases of barbiturate poisoning. Rosendorff (1910), Sowden (1910) and MacLean (1912) found no abnormal porphyrinuria in patients who had ingested single large doses of diethylbarbituric acid, while Rommel (1912) observed dark-red urine in a patient who had taken 25 g. of

the same drug. Lehmann & Zinn (1910) have described a urinary pigmentation, suggestive of porphyrin and porphobilin, in a patient who had received 0·5 g. diethylbarbituric acid. They gave this drug in prolonged dosage to one rabbit without the production of similar pigments. Animal experiments were also carried out by Laubender & Monden (1938), who investigated the effects of diethyl, bromallyl*iso*propyl and ethyl*cyclo*hexenylbarbituric acids on the urinary porphyrins of rabbits. They failed to find any such effect in all these experiments with the exception of one animal treated with diethylbarbituric acid, which showed a slight rise of urinary porphyrins.

The barbiturates comprise a group of chemically related drugs, while acute porphyria is a rare disease. Moreover, it is usually impossible to tell from published records whether the barbiturates had precipitated the overt disease in a case of latent porphyria or had in fact provoked acute porphyria in a formerly healthy subject. To overcome this difficulty and the uncertainty in assessment of these clinical impressions, the effects of different barbiturates on the porphyrin metabolism of rabbits were studied. Out of nine barbiturates so tested, six had a significant effect on the urinary porphyrin excretion; those containing one or more allyl groups were particularly effective.

METHODS

Seventeen rabbits (average wt. 2·8 kg.) were housed in individual metabolism cages allowing the separation of urine and faeces. A preliminary base-line period of 4–7 days before drug administration was allowed during which the total daily urinary coproporphyrin and sometimes also faecal porphyrins were determined. If the animal died during an experiment, tissue porphyrins and porphobilinogen were also determined. The average duration of each course of barbiturate was 12 days. The types of barbiturates and their route of administration are recorded in Table 1.

Table 1. *Effect of barbiturates on the urinary excretion of coproporphyrin in rabbits*

The sodium barbiturates were given in equally divided dosage twice daily. A letter after a rabbit number signifies that the rabbit obtained the corresponding barbiturate on a primary course *a*, or on subsequent courses *b*, *c*, etc. Doses were administered by intramuscular injection unless indicated otherwise.

		Dosage		Mean urinary coproporphyrin	
Drug	Rabbit no.	Average level (mg./kg./day)	Duration (days)	Pre-dosage level (μg./day)	Increase (μg./day)
5:5-Diallylbarbituric acid	2*a*	113*	15	19·7	+193·7
	1*b*	103*	35	7·5	+169·5
	3	100	17	5·2	+119·2
	4*a*	128*	12	8·7	+88·3
	5	101·6	7	8	+86
	6	125	9	5·3	+84
	7	114	7	2	+51
	8	148	7	3·3	+47
Sodium 5-allyl-5-(1-methylbutyl)-barbiturate	9	172	7	3·7	+42·6
	10	115	12	3·8	+22·9
	11*b*	100	12	9·8	+20·2
	2*d*	82·8	12	19·7	+17·3
5-Allyl-5-*iso*propylbarbituric acid	2*b*	67·8†	12	19·7	+81·6
	12	76†	13	7	+40
	13	74†	12	7	+27
Sodium 5:5-diethylbarbiturate	14*b*	171	13	5	+ 9·7
	13*d*	140	12	7	+ 9·6
	15	169	13	1·8	+ 8·7
	16	144	12	2·4	+ 5·6
Sodium 5-ethyl-5-(1-methylbutyl)-barbiturate	11*a*	98·5	12	9·8	+15·2
	2*c*	110·5	12	19·7	+ 7·3
	4*b*	128	14	8·7	+ 2·3
Sodium 5-ethyl-5-phenylbarbiturate	14*a*	71	9	5	+14·7
	1*a*	67	20	7·5	+13·5
	13*c*	81	12	7	+11·7
	16*b*	82	12	2·4	+ 6·8
Sodium 5-*iso*amyl-5-ethylbarbiturate	13*b*	90	12	7	− 2
	2*e*	83	12	19·7	− 5·2
Sodium 5-butyl-5-ethylbarbiturate	17*a*	109·5	12	11·5	+ 5·5
	16*a*	98	12	2·4	+ 2·3
Sodium 5-ethyl-5-(1-methylbutyl)-2-thiobarbiturate	17*b*	218	12	11·5	+ 5·2
	2*f*	164	12	19·7	− 1·7

* Some doses given by gastric intubation. † Given by gastric intubation.

If the rabbit survived this course, analyses were continued during a recovery period of 4–7 days. After such an interval, some rabbits were started on a different barbiturate as indicated in Table 1. The maximum sub-lethal dose of each drug was employed.

Quantitative analyses

Urinary porphyrins. These were determined by the method of Rimington & Sveinsson (1950) with the following modifications. Ether-soluble porphyrins were extracted by ether: acetic acid (10:1 by vol.) and after the ether had been washed three times with water, were transferred directly into 1·4 N-HCl for spectrophotometric determination (Beckman, model DU). One part of urine containing porphobilinogen, was diluted with 4 parts 2 N sodium acetate buffer pH 4·22, and placed in boiling water for 20 min. Appropriate dilution for spectrophotometric determination of the total uroporphyrin was made with HCl so that the final concentration of acid was 0·5 N.

Faecal coproporphyrin and tissue copro- and proto-porphyrin. Faeces and tissues were ground thoroughly in a mortar. Liver tissue was in addition comminuted in a Waring Blendor. The ether-soluble porphyrins from weighed portions of faeces and tissue were extracted by repeated shaking with ether: acetic acid (10:1, v/v) until the supernatant after centrifuging showed no porphyrin fluorescence. The porphyrins were then analysed as in the case of urine.

Porphobilinogen. Porphobilinogen in urine was determined by the method of Vahlquist (1939). Tissue dispersions were treated with about an equal volume of 20% (w/v) trichloroacetic acid, the mixture was centrifuged and the supernatant analysed as in the case of urine.

Blood examination. Haemoglobin was determined by the method of Rimington (1942). Erythrocyte counts and reticulocyte counts were carried out using standard techniques.

Urinary amino acids. These were examined by methods of Datta, Dent & Harris (1950).

Identification of porphyrins

The urines from rabbits treated with sodium allyl(1-methylbutyl)barbiturate or allyl*iso*propylbarbituric acid contained increased amounts of porphyrins, all of which were ether soluble. These urines were preserved with toluene and stored at 3°. The porphyrins were extracted with ether: acetic acid and, after the ether had been washed 3 times with water, were transferred into a minimal volume of 2·8 N-HCl and then precipitated at the isoelectric point (pH 3·1). The precipitate was centrifuged, dried, and esterified by contact with methanolic HCl for 24 hr. The porphyrin esters were transferred into $CHCl_3$ by the addition of saturated sodium acetate, the $CHCl_3$ layer washed once with water, once with dilute ammonia, and twice with water, in that order. The α band (absorption band at longest wavelength) of the porphyrin ester at this stage was noted on the Hartridge Reversion Spectroscope. A portion of the ester was dried, hydrolysed with 7 N-HCl for 36 hr. and chromatographed on paper (Nicholas & Rimington, 1951). The remainder was crystallized from $CHCl_3$: methanol (1:5, v/v). Melting points, which were not corrected, were determined on an electrically heated micro apparatus (A. Gallenkamp and Co. Ltd.). Isomer analysis was done by the method of Chu, Green & Chu (1951).

Urines from some rabbits treated with diallylbarbituric acid contained uroporphyrin and porphobilinogen as well as an increase in ether-soluble porphyrins. These combined urines were brought to pH 3–4 with glacial acetic acid and sufficient talc was added to adsorb all porphyrins. The talc was filtered, dried, and repeatedly eluted with 2 N ammonia until no porphyrin fluorescence appeared in the supernatant. The total alkaline eluates were then brought to the isoelectric point and the porphyrins thus precipitated were dried, esterified and taken into $CHCl_3$ as described above. The porphyrin esters were fractionated by chromatography on columns of aluminum oxide Grade IV and magnesium oxide Grade III (Nicholas, 1951). The fractions were defined by α band estimations and, after hydrolysis, by paper chromatography (Nicholas & Rimington, 1951). Isomer analysis of octacarboxylic porphyrin esters was performed by the method of Falk & Benson (1953), while that on tetra- and penta-carboxylic porphyrins was done by the method of Chu *et al.* (1951). The uroporphyrin ester was crystallized from chloroform: methanol.

RESULTS

Those barbiturates which produced the greatest increase of urinary porphyrins also caused the most prolonged hypnosis. Diallylbarbituric acid was the most effective drug, while allyl*iso*propylbarbituric acid and sodium allyl(1-methylbutyl)barbiturate, caused a higher excretion of urinary coproporphyrin and a more prolonged hypnosis than the remaining barbiturates. In each of three rabbits treated with diallylbarbituric acid, the drug was given sometimes by gastric intubation and sometimes by intramuscular injection. No significant quantitative difference of urinary porphyrin excretion was obtained by thus changing the route.

Urinary coproporphyrin. Table 1 records the average daily excretion of coproporphyrin in the urine during the pre-dosage period and the average deviation from this figure during administration of the drug. Coefficients of variation are not included since in almost every instance the results were quite obviously significant. Where an effective barbiturate was used, the urine coproporphyrin excretion showed a prompt increase after the 1st or 2nd day, rose to a maximum level on about the 6–7th day, remained at about that level during the subsequent administration of the drug and fell to normal within 2–3 days of its cessation.

Urinary uroporphyrin and porphobilinogen. In three rabbits (1*b*, 2*a* and 3) treated with diallylbarbituric acid, uroporphyrin and porphobilinogen appeared in the urine on the 7th, 7th and 8th days, respectively, and generally continued to be excreted for as long as the drug was given (Table 2 and Fig. 1).

Faecal coproporphyrin. In rabbit 1*b* the faecal coproporphyrin was determined before and during administration of diallylbarbituric acid. This showed that the faecal coproporphyrin rose at the same rate as the urine coproporphyrin from a

previous normal daily average of 38 μg./day to a daily average of 1495 μg./day.

Tissue porphyrins. Table 3 summarizes the tissue analyses for porphyrin of four normal rabbits, and five rabbits which had died from barbiturate intoxication at a stage when they were excreting increased urinary coproporphyrin. There was an increase of copro- and proto-porphyrin concentrations in the bile and liver in intoxicated rabbits, but the levels in the bone marrow showed no abnormality. The liver of rabbit 10 gave a faint but definitely positive reaction for porphobilinogen. Uroporphyrin was never found in any tissue.

Blood examination. Haemoglobin determinations, erythrocyte counts and reticulocyte counts were made immediately before, during and after administration of the drug to three rabbits treated with diallylbarbituric acid and one treated with allyl*iso*propyl barbituric acid. No significant change was observed in any of the erythrocyte counts or reticulocyte counts. In two of these rabbits (2*b* and 3), a slight fall (about 2 g. %) in the haemoglobin level was observed, while two rabbits (1*b* and 7) showed no significant change.

Table 2. *Daily urinary uroporphyrin and porphobilinogen excretion in rabbits receiving diallylbarbituric acid*

Rabbit no.	Total uroporphyrin (μg./day) Average	Maximum	Porphobilinogen (units*/day) Average	Maximum
1*b*	1720	4750	456	1607
2*a*	360	800	78	161
3	250	340	78	109

* Vahlquist (1939).

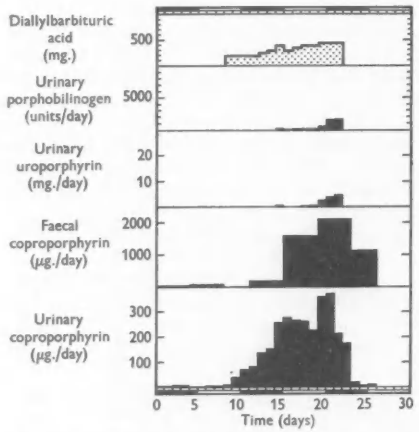

Fig. 1. Effects of diallylbarbituric acid on the urinary uroporphyrin, coproporphyrin and porphobilinogen and faecal coproporphyrin excretion of rabbit 1*b*.

Table 3. *Tissue porphyrin determinations on four normal rabbits and five rabbits treated with barbiturates*

The five rabbits died while excreting excessive urinary coproporphyrin. Figures represent μg./ml. (bile) or μg./g. (liver and marrow).

Rabbit no.	Bile		Liver			Bone marrow		
	Copro-porphyrin	Proto-porphyrin	Copro-porphyrin	Proto-porphyrin	Porpho-bilinogen	Copro-porphyrin	Proto-porphyrin	Porpho-bilinogen
Normal								
Control I	0·15	Nil	0·10	0·10	Nil	0·10	Nil	Nil
Control II	0·38	0·34	0·18	0·12	Nil	0·20	0·15	Nil
Control III	0·74	0·41	0·18	0·51	Nil	0·22	0·24	Nil
Control IV	Insufficient obtained		0·02	0·23	Nil	0·18	0·16	Nil
Diallylbarbituric acid								
8	Insufficient obtained		5·5	49	Nil	0·10	0·12	Nil
7	25	172	11·8	30·9	Nil	0·12	0·14	Nil
5	5·2	84·0	3·2	4·66	Nil	0·15	0·22	Nil
Sodium allyl(1-methylbutyl)barbiturate								
10	0·78	12·68	4·76	17	Faint +	0·10	0·24	Nil
9	Insufficient obtained		1·91	3·84	Nil	0·30	0·31	Nil

Rabbit no.	Uroporphyrin Bile	Liver	Bone marrow
Normal			
Control I	Nil	Nil	Nil
Control II	Nil	Nil	Nil
Control III	Nil	Nil	Nil
Control IV	Nil	Nil	Nil
Diallylbarbituric acid			
8	Nil	Nil	Nil
7	Nil	Nil	Nil
5	Nil	Nil	Nil
Sodium allyl(1-methylbutyl)barbiturate			
10	Nil	Nil	Nil
9	Nil	Nil	Nil

Table 4. *Identification of urinary porphyrins. Summary of results*

	Free porphyrin	Porphyrin ester				
			Paper chromatography			
	Paper chromatography (Nicholas & Rimington, 1951)	α Band	(*a*) Chu *et al.* 1951	(*b*) Falk & Benson, 1953	M.p. of crystals (°)	Summary
Diallylbarbituric acid	8 COOH	625·1	—	Mainly series III	274	Uroporphyrin III
	6 COOH	623·6	—	—	—	Hexacarboxylic porphyrin
	5 COOH	622·5	Series III	—	—	Pentacarboxylic porphyrin III
	4 COOH	621·1	Series III	—	—	Coproporphyrin III
Sodium allyl(1-methylbutyl)barbiturate	4 COOH	621·3	Mainly series III	—	154, remelt. 172–4	Coproporphyrin III
Allyl*iso*propylbarbituric acid	4 COOH	621·7	Mainly series III	—	150, remelt. 160	Coproporphyrin III

Urinary amino acids. In seven rabbits, in which a rise of urinary coproporphyrin was obtained, the urinary amino acid pattern was no different before and at the end of barbiturate administration.

Identification of porphyrins. The ether-soluble porphyrins obtained from the urines of rabbits treated with allyl*iso*propylbarbituric acid or sodium allyl(1-methylbutyl)barbiturate consisted entirely of coproporphyrin III. The ester m.p.'s and the results of paper chromatograms are shown in Table 4.

The porphyrin esters obtained from the urine of rabbits treated with diallylbarbituric acid were fractionated on a magnesium oxide column. This separated first an octacarboxylic porphyrin with an α band 625·0 mμ.; then two intermediate fractions with α bands 623·0 and 621·6 mμ., respectively; and finally a tetracarboxylic porphyrin with α band 621·1 mμ. The first fraction was rechromatographed on a magnesium oxide column and yielded a single porphyrin zone. The appearance of the crystals obtained from this zone was suggestive of uroporphyrin III. The m.p. (274°) and paper chromatography (Falk & Benson, 1953) showed the series III isomer to be that mainly present (see Table 4). The two intermediate fractions were further chromatographed on an aluminium oxide column. This allowed the separation of a main portion with α band 622·5 mμ. and a lesser portion with α band 623·5 mμ., which behaved as pentacarboxylic and hexacarboxylic porphyrins, respectively, when examined by paper chromatography (Nicholas & Rimington, 1951). On a paper chromatogram (Chu *et al.* 1951), the pentacarboxylic porphyrin had about the same mobility as coproporphyrin III. The final fraction was identified as coproporphyrin III by melting point and paper chromatography (see Table 4).

DISCUSSION

This work has shown that of the nine different barbiturates examined, six were capable of producing some effect on the porphyrin metabolism of rabbits. Of seventeen rabbits used, eight were given more than one barbiturate in separate courses. This was considered as a possible factor influencing results. However, another barbiturate was never started until the urinary coproporphyrin level had returned to its normal from the previous course, and the results in Table 1 show that the same drug, used on different animals, whether it was their first or subsequent barbiturate, generally gave corresponding results in porphyrin excretion. For the more effective barbiturates, sufficient numbers of rabbits were used, on a primary or subsequent barbiturate course, to demonstrate the unimportance of previous intoxication. Difference in effect upon porphyrin excretion must depend, among other things, upon the chemical constitution of the drug, the daily dosage employed and its duration, and also, possibly, upon the normal level of porphyrin excretion of the individual animal.

The results in Table 1 show that the barbiturates used may be classified in four groups according to their effectiveness (Fig. 2).

(1) Diallylbarbituric acid, which caused a considerable rise of urinary coproporphyrin in each of eight rabbits and, in three of these, the excretion also of uroporphyrin and porphobilinogen.

(2) Allyl*iso*propylbarbituric acid and sodium allyl(1-methylbutyl)barbiturate, which caused a moderate rise of urinary coproporphyrin.

(3) Sodium diethylbarbiturate, sodium ethyl(1-methylbutyl)barbiturate, and sodium ethylphenylbarbiturate, which caused only a slight increase of coproporphyrin in the urine.

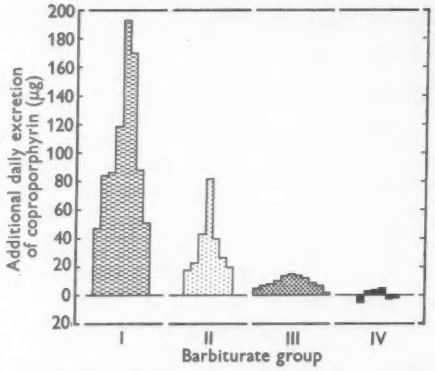

Fig. 2. Each vertical segment represents the average daily coproporphyrin output in a single rabbit during barbiturate administration in excess of (or less than) the normal average daily coproporphyrin excretion. I, diallylbarbituric acid; II, allyl*iso*propylbarbituric acid and sodium allyl(1-methylbutyl)barbiturate. III, sodium diethylbarbiturate, sodium ethyl(1-methylbutyl)barbiturate, and sodium ethylphenylbarbiturate. IV, sodium ethyl(1-methylbutyl)thiobarbiturate, sodium *iso*amylethylbarbiturate, and sodium butylethylbarbiturate.

(4) Sodium ethyl(1-methylbutyl)thiobarbiturate, sodium *iso*amylethylbarbiturate, and sodium butylethylbarbiturate, which did not alter significantly the urinary coproporphyrin excretion.

The possession of the allyl group thus appeared to make the barbiturate more effective in producing a rise of porphyrin in the urine. These drugs also caused deeper and more prolonged hypnosis in rabbits. Drugs possessing an allyl group have been known to give rise to pathological and metabolic changes in humans and in experimental animals. Popper (1936) and Jürgens (1951) have shown that allyl formate and *N*-(diallylacetyl)urea, respectively give rise to hepatic cellular degeneration in experimental animals. *N*-(Allyl*iso*propylacetyl)urea may cause thrombocytopenic purpura in humans (Ackroyd, 1949), may influence the course of human acute porphyria (Duesberg, 1932), and gives rise to the hepatic type of experimental porphyria in rabbits with the excretion of large amounts of uroporphyrin and porphobilinogen (Schmid & Schwartz, 1952). Diallylbarbituric acid has for some time been known to be one of the most toxic of the barbiturates and for this reason has generally been abandoned as an hypnotic in humans.

The finding in post-mortem tissue analysis that the liver and not the bone marrow contained excessive copro- and proto-porphyrin (and in one instance porphobilinogen) recalls the finding in the liver of porphobilinogen (Prunty, 1945) and uroporphyrin (Watson, Schwartz & Hawkinson, 1945) in human acute porphyria and of these two substances in experimental porphyria in rabbits (Schmid & Schwartz, 1952). It also may be related to the known hepatotoxic effect of other allyl derivatives in experimental animals. It is of interest, too, that the coproporphyrin and uroporphyrin produced in excess were the series III isomers. Rimington (1952) has proposed a scheme of possible enzymic derangements, occurring in the porphyrias and the toxic porphyrinurias. It is not possible from the present results to interpret the precise mechanism occurring in the barbiturate porphyrinurias, but a reasonable inference from them might be that some of the barbiturates, especially those containing the allyl group, may interfere with the enzymes which decarboxylate uroporphyrin III to coproporphyrin III and protoporphyrin and, perhaps, with those responsible for the transformation of protoporphyrin to haem. The main site of this interference may possibly be the liver.

The present findings raise the questions of the bearing of these animal experiments upon barbiturate medication in human beings and of the relation, if any, which exists between human acute porphyria and the disturbances of pigment metabolism caused by barbiturates in rabbits. The doses of barbiturates used in the animal experiments were proportionately (weight for weight) greatly in excess of those normally taken by humans. The rabbits, however, generally tolerated these daily doses well and were mostly able to survive the period of drug administration. Humans if given a proportionate amount of barbiturate almost certainly could not survive. A weight-for-weight comparison of the effect of the barbiturates on human and rabbit porphyrin metabolism would therefore be misleading. From these experiments, we may possibly infer that barbiturates in proportionately smaller dosage may affect the abnormally sensitive porphyrin metabolism in human acute porphyrias; the clinical impression that this does in fact take place may receive some substantiation from these results. The differing degree of effectiveness of the barbiturates in the experimental animal may also explain the divergence of opinion on this clinical impression, which was based on the observation of different barbiturates. It is of great interest that diallylbarbituric acid administered to a normal animal causes the excretion of porphobilinogen and uroporphyrin.

A further point of inference may be important. Barbiturates have been shown to inhibit some of the oxidative processes of brain tissue (Quastel & Wheatley, 1933), the degree of inhibition being directly proportional to the hypnotic activity of the drugs. These authors also emphasized the importance of the allyl group in this connexion. Changes in

nerve and brain tissue occurring in acute porphyria (Mason, Courville & Ziskind, 1933; Denny-Brown & Sciarra, 1945) may be the most important lesions responsible for the clinical results of this disease. It is possible that barbiturates might aggravate this upset in brain and nerve metabolism, as distinct from their effect on porphyrin metabolism. This might explain why some barbiturates, producing no marked effect on the porphyrin metabolism of rabbits, have been among those suspected of adversely affecting the course of acute porphyria.

SUMMARY

1. Barbiturates have been suspected of adversely affecting the clinical course of acute porphyria. To assess this clinical impression objectively, the effect of nine barbiturates on the porphyrin metabolism of rabbits has been observed.

2. These barbiturates may be classified into four groups. (i) Diallylbarbituric acid, which caused a considerable rise of urinary coproporphyrin III in each of eight experimental rabbits and in three of these the excretion also of uroporphyrin III and porphobilinogen. (ii) Allyl*iso*propylbarbituric acid and sodium allyl(1-methylbutyl)barbiturate, which caused a moderate rise of urinary coproporphyrin III. (iii) Sodium diethylbarbiturate, sodium ethyl-(1-methylbutyl)barbiturate and sodium ethylphenylbarbiturate, which caused only a slight coproporphyrin increase in the urine. (iv) Sodium ethyl(1-methylbutyl)thiobarbiturate, sodium *iso*amylethylbarbiturate and sodium butylethylbarbiturate, which did not alter significantly the urinary coproporphyrin excretion.

3. The effect of the presence of an allyl group in the barbiturate is discussed.

4. The significance of these results in animals is considered in relation to human acute porphyria.

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Distribution of Enzymes in Cell-Free Yeast Extracts

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The evidence for the existence of 'mitochondria' in micro-organisms has been growing, but the criteria for thus naming cytoplasmic inclusions are by no means clear. From the biochemical point of view, mitochondria are generally considered to be sites of oxido-reduction and of organized oxidations. The 'cyclophorase' complex in the mitochondria of animal cells studied by Green and his School (Green, Loomis & Auerbach, 1948) has never been demonstrated in micro-organisms, although it has recently been found in plants (Millerd, Bonner, Axelrod & Bandurski, 1951).

The failure to prepare cell-free, actively respiring yeast extracts contributes to the continuing controversy about the main respiratory mechanism of this micro-organism (cf. Krebs, Gurin & Eggleston, 1952; Martius & Lynen, 1950; and Foulkes, 1951).

Methods used so far for preparing yeast extracts are probably such that the intracellular organization of enzymes, and therefore organized oxidation systems, are destroyed during cell disintegration. We have developed an ultra-rapid mechanical shaker which disintegrates 2 g. yeast almost entirely within 90 sec. (Nossal, 1953*a*). We failed to prepared actively respiring yeast 'mitochondria' with this machine. However, the results reported here show that the distribution of enzymes in the cell-free extracts is not unlike that in extracts of animal cells: dehydrogenases are found largely associated with the granular fraction when the extracts are prepared by very brief disintegration.

While the work was in progress, Hirsch (1952) reported that fumarase and aconitase were found largely in the non-sedimentable supernatant obtained after spinning yeast extracts at 30 000 *g*. Hirsch's extracts were prepared with a Towers Ultra-Rapid Shaker at 560 rev./min. The shaker used here disintegrates micro-organisms about 100 times more rapidly. Hirsch's findings were therefore re-examined in the light of the results obtained for dehydrogenases. A preliminary report of the very high fumarase and aconitase activities of the granules in cell-free extracts, prepared by 10 sec. disintegration in our shaker, has appeared elsewhere (Nossal, 1953*b*).

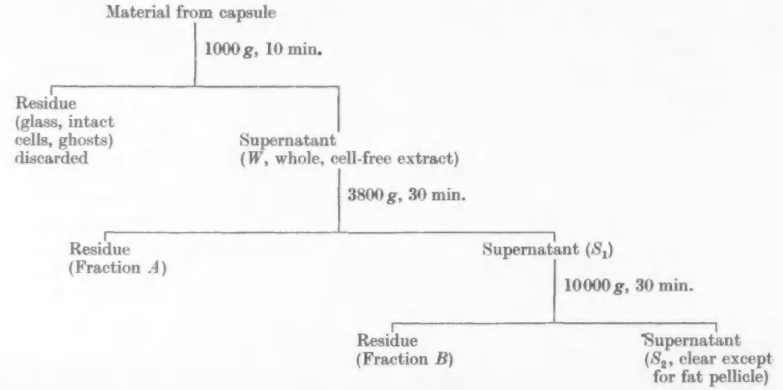
METHODS

Materials. Baker's yeast was obtained weekly from the Effront Yeast Co., South Yarra, Victoria, and kept in a humidor at 1°. Cakes may be kept thus without decolorization or loss of respiratory activity for up to 4 weeks.

Chemicals used were commercial samples, except 80% pure diphosphopyridine nucleotide (DPN), prepared from yeast by the method of Kornberg & Pricer (1951), and triphosphopyridine nucleotide (TPN) from liver (Kornberg & Horecker, private communication), either 10% pure and containing 9% DPN or 50% pure and free of DPN.

Mechanical disintegration was carried out as described previously (Nossal, 1953*a*): the stainless steel shaker capsule was charged with 2 g. wet wt. yeast, 10 g. Ballotini glass beads no. 12 (The English Glass Co., Leicester, England) and 10 ml. 0·9% KCl, cooled to 0° and shaken at 5600 rev./min. with an amplitude of $\frac{3}{8}$ in. (maximum acceleration about 300 *g*) for 10, 30 or 90 sec. at 1°.

Fractionation of the extract was effected by differential centrifugation as follows:


Material from capsule
1000 g, 10 min.
Residue (glass, intact cells, ghosts) discarded
Supernatant (W, whole, cell-free extract)
3800 g, 30 min.
Residue (Fraction A)
Supernatant (S_1)
10000 g, 30 min.
Residue (Fraction B)
Supernatant (S_2, clear except for fat pellicle)

W was examined routinely and found to contain less than 0·1% of the original cells. This represents a negligible proportion when compared with disintegrated cells (see Table 2). The particulate fractions were washed once with as much 0·9% KCl as the original volume of *W*, unless otherwise stated. Dry weights were obtained by heating 2 ml. portions to 120° for 3 hr.

All manipulations were carried out as near 0° as possible, but at the end of 90-sec. disintegrations, the temperature of the capsular contents was about 25°; the capsular contents were cooled immediately. Dialyses were carried out at 0° for 15 hr., the dialysis sac being attached to a small magnet and rotated on a magnetic stirrer.

Activity measurements. Dehydrogenase activities were measured *in vacuo* in Thunberg tubes at 38°. The tubes contained 0·02 M-Na_2HPO_4/KH_2PO_4 buffer pH 7·0, 0·1 M substrates (neutralized if necessary), 10^{-4} M methylene blue, other additions as indicated and water to give a total fluid volume/tube of 3·0 ml.

Activities are expressed as $Q_{M.B.}$ values, which were calculated from 90% decolorization times. $Q_{M.B.} = \mu$l. methylene blue decolorized/mg. dry wt./hr. In some of the systems, the decolorization times were not linearly proportional to the amount of enzyme present. Therefore, for the purposes of comparison, equal amounts (on a dry wt. basis) of various extracts and fractions were taken for each experiment. Otherwise, the mean values obtained from several sets of experiments are cited.

Aconitase and fumarase activities were measured as rates of increase in optical density at 240 mμ. with citrate and L-malate, respectively, as substrates (Racker, 1950). The assay system contained enzyme, 0·01 M phosphate buffer pH 7·0, 0·01 M substrate and glass-distilled water to a final volume of 3·0 ml. Blanks contained no substrate. Room temperature was approx. 20°. No attempt was made to regulate the temperature in the spectrophotometer, but the temperature variation over a whole day was less than 2°.

For fumarase, the rate of change of optical density was directly proportional to enzyme concentration over a wide range (up to 0·1 density change/min.). For aconitase, the linearity held only for lower activities (up to 0·015/min.). Also, whereas for fumarase the rate of change of optical density was constant for at least 15 min., in the case of aconitase it declined after about 5 min. Therefore, specific activities (increases in optical density/mg. dry wt./min. $\times 1000$) were calculated from the mean of the first ten 30-sec. readings (0·5–5·5 min. after mixing) for both enzymes. Activities reported here, multiplied by 10, should be comparable with those of Hirsch (1952), whose results are expressed on a mg. protein N basis.

At first, measurements were made in 0·9% KCl to preserve granular structures in the extracts (under the phase contrast microscope the granules were better defined in 0·9% KCl than in water). It was found that this concentration of KCl inhibited fumarase activity up to 50% in all fractions. Aconitase was much less affected. Similar inhibition was observed when KCl was omitted and the buffer concentration increased to 0·1 M. Accordingly, all subsequent dilutions in the test system were made with glass-distilled water. The total salt concentration during measurements (including enzyme, buffer and substrate) was maintained below 0·04 M.

RESULTS

Activities in whole, dialysed extracts. Without dialysis, *W* and S_2 had high blank dehydrogenase activities. All results for dehydrogenases quoted in this work were obtained with dialysed *W* and S_2 or washed granules. The highest blank activities ($Q_{M.B.}$ values occasionally up to 4) were observed in the presence of impure TPN, containing DPN, in 30-sec. whole extracts. Otherwise the blank $Q_{M.B.}$ values were less than 1.

The dehydrogenase activities studied depended to some extent on the disintegration period used in the preparation of the extracts (Table 1). The ethanol, malic and glutamic dehydrogenases were little affected by the duration of disintegration, but the succinic, 'citric' and lactic dehydrogenase activities were much lower in 90-sec. extracts than in 10- or 30-sec. extracts.

Table 1. *Dehydrogenase activities in whole, dialysed yeast extracts*

Cell-free extracts were prepared by concurrent 10-, 30- and 90-sec. disintegrations from the same batch of yeast. 90% decolorization of methylene blue was measured as described under Methods. Dialyses were for 15 hr. against 50 vol. 0·9% KCl at 1°. $Q_{M.B.}$ values (μl. methylene blue decolorized/mg. dry wt./hr.) are the means of four complete series of experiments.

		$Q_{M.B.}$		
		10-	30-	90-
Substrate	Other additions	sec. disintegration		
None	0·1 mg. DPN 0·1 mg. TPN	<1	<4	<0·5
Succinate	—	6·6	7·1	<2·5
Citrate	0·1 mg. TPN 0·1 mg. DPN 0·001 M-$MnCl_2$ 0·001 M Adenosine 5-phosphate	8·7	10	2·8
Ethanol	0·1 mg. DPN	65	65	65
Lactate	—	20	19	10
L-Malate	0·1 mg. DPN	30	28	24
L-Glutamate	0·1 mg. TPN	17	18	14

Table 2. *Effect of disintegration period on the dry-weight distribution in cell-free yeast extracts*

Values given represent means of 20–40 samples for each disintegration period. The percentage disintegration was determined by direct counting of intact cells and ghosts on a haemocytometer at 400-fold magnification.

Disintegration period (sec.)	% disintegration	Fraction	Dry wt. (mg./ml. original extract)	% of dry wt. of whole extract
10	35	*W*	14·4	100
		Particulate, *A*	1·1	7·6
		Particulate, *B*	1·2	8·3
		S_2	11·3	78
		Combined washings*	0·8	5·6
30	60	*W*	22·1	100
		Particulate, *A*	2·4	11
		Particulate, *B*	2·7	12
		S_2	16·4	74
		Washings*	1·5	6·8
90	85	*W*	31·7	100
		Particulate, *A*	2·8	8·8
		Particulate, *B*	5·3	17
		S_2	21·7	68
		Washings*	2·5	7·9

* Sum of dry weights of 1 ml. each of the washings from particulate fractions *A* and *B*.

The activities given for citrate in *W*, S_2 and particles are much lower than the *iso*citric dehydrogenase content of these systems. The reason for using citrate as a substrate was the non-availability of *iso*citrate in sufficient amounts. The dehydrogenase activities measured therefore depended also on aconitase. This enzyme is notoriously unstable, especially to dialysis (see Hirsch, 1952). With the small amount of *iso*citrate available, it was shown that low concentrations of *iso*citrate gave much greater activities than high concentrations of citrate. With 0·01 M *iso*citrate, the highest $Q_{M.B.}$ value was 53 for a whole extract. This is in accordance with the high rates of citrate breakdown in yeast extracts reported by Foulkes (1951) and Hirsch (1952), and shows that *iso*citric dehydrogenase is one of the most active enzymes present in the extracts.

Effect of disintegration period on the relative proportions of particulate fractions. Examination of fresh yeast cells under phase contrast showed cytoplasmic granules with high refractive index and fairly uniform size. Similar findings were reported recently by Sarachek & Townsend (1953). It seemed possible that fraction *B* of yeast extracts was formed by mechanical disintegration of fraction *A*. The percentage of *B* should then increase with increasing disintegration period. This was found experimentally. 10- and 30-sec. extracts contained approximately equal amounts of fractions *A* and *B* (Table 2). In 90-sec. extracts, the sum of particulate fractions approximately equalled that in 30-sec. extracts, but only one-third of the material sedimented at the low speed and two-thirds at the higher speed. The increased weight of 30- and 90-sec. combined particles over 10-sec. combined particles was probably due to smashed cell-wall fragments not removed by centrifuging at 1000 *g*.

Effect of disintegration period on the enzymic activities of particles. While this work was in progress, Slonimsky & Hirsch (1952) reported in a brief note that the following enzymes predominated in the non-sedimentable supernatant of cell-free yeast extracts: lactic, malic, ethanol and *iso*citric dehydrogenases, fumarase and aconitase.

The dehydrogenase activities of granules in yeast extracts were here found to depend on the disintegration period employed in preparing the extract (Table 3). The particulate fractions of 10-sec. extracts had very high ethanol, malic and succinic dehydrogenase activities and also appreciable activities of the other enzymes. All activities were much lower in 90-sec. particles, the decreases being least for succinic dehydrogenase and greatest for ethanol and malic dehydrogenases. Glutamic and lactic dehydrogenases seemed to predominate in the supernatant. At present it is not possible to ascertain whether these enzymes really do occur mainly free in the cytoplasm of the intact cell, or whether they are the most easily removed from cytoplasmic granules. The extracts prepared by Hirsch (1952) and Slonimsky & Hirsch (1952) were made on a Towers Ultra-Rapid Shaker, which is about 10 times less rapid than ours. It is probable that after the much longer disintegration periods which had to be employed (60 min. as against 10 sec.), the more soluble enzymes appear largely in the supernatant. Even by a single washing with 0·9 % potassium chloride, particulate fractions lost weight and some dehydrogenase activities, which were recovered in the washing solution. Hirsch's disintegration medium was 0·1 M phosphate buffer, pH 7·3, and in 60 min. considerable amounts of dehydrogenases would be detached from the particles.

Table 3. *Effect of disintegration period on dehydrogenase distribution*

Washed particulate fractions *A* and *B* were prepared from 10-, 30- and 90-sec. extracts as described under Methods. Additions for each substrate as in Table 1. $Q_{M.B.}$ values are the means of the number of experiments shown.

Disintegration period (sec.)	Particulate fraction	No. of experiments	$Q_{M.B.}$ Blank	Succinate	Citrate	Ethanol	Lactate	L-Malate	L-Glutamate
10	*A*	3	<0·5	21	7*	49	9	27	5
	B	3	<0·5	14	3	33	7	33	1
30	*A*	9	<0·5	16	3	16	8	15	2
	B	6	0·4	16	3	23	8	14	1
90	*A*	8	<0·5	10	1	9	4	9	0·5
	B	5	<0·5	7	<0·5	5	3	5	<0·5

* The highest activity with 0·01 M *iso*citrate was 26.

Table 4. *The effect of disintegration period on the distribution of fumarase and aconitase in cell-free yeast extracts*

The specific activities (increase in optical density at 240 mμ./mg. dry wt./min. × 1000) are the means of three complete series for each disintegration period.

Disintegration period (sec.)	Activity measured	Specific activities of the fractionated extract: Whole extract	Particulate fraction *A*	S_1	Particulate fraction *B*	S_2	Washings of fraction *A*	Washings of fraction *B*
10	Fumarase	109	130	116	155	126	70	93
30	Fumarase	131	39	157	64	199	89	152
90	Fumarase	135	7	145	6	212	102	138
10	Aconitase	37	16	34	27	39	18	15
30	Aconitase	41	8	49	14	62	31	43
90	Aconitase	49	3	49	3	74	34	39

Table 5. *Effect of double washing on the enzymic activities of yeast granules*

10-sec. whole extracts were centrifuged at 10000 *g* to separate them into particles (fractions *A* plus *B*) and S_2. The particles were washed as indicated below. Activities refer to those of the combined particulate fraction. $Q_{M.B.}$ values are the means of two separate series, specific fumarase and aconitase activities means of three series.

Washing solution	No. of washings	$Q_{M.B.}$ Succinate	Citrate	Ethanol	Lactate	L-Malate	Specific activities: Fumarase	Aconitase
0·9% KCl	1	17	1·6	52	8	19	121	30
0·9% KCl	2	15	2·3	47	9	21	162	29
Water	1	11	4·7	50	6	18	110	42
Water	2	17	5·9	58	7	18	147	51

The diminished dehydrogenase activities of 30- and 90-sec. particles were largely accounted for by correspondingly increased activities in the supernatants, except for those enzymes which seemed to be denatured by prolonged disintegration (Table 1). Succinic dehydrogenase was found in 90-sec. supernatants and in the washings of 90-sec. granules. Both findings point to extensive disorganization of the particles.

The results for fumarase and aconitase were similar: the particles of 10-sec. extracts had fumarase activities at least as high, and occasionally twice as high, as those of the supernatant (Table 4). Their aconitase activity was usually lower than that of the supernatant. 30-sec. particles had fumarase activities between 25 and 50% of those of the corresponding supernatants, and still lower aconitase activities. The fumarase and aconitase activities of 90-sec. particles were less than 10% of those of the supernatant and often too low to measure.

Both particulate fractions from all extracts had a very similar enzymic composition. This, coupled with the probable conversion of fraction *A* into fraction *B* by prolonged mechanical disintegration, suggests that the fractions do not represent functionally or morphologically different particles, but that there is one type of granule, rich in respiratory enzymes, in yeast cytoplasm.

To rule out the possibility that the observed granular dehydrogenase and hydrase activities were due to contamination of the granules with supernatant, the effect of a second washing on the

activities of particulate fractions was examined. Both water and 0·9 % potassium chloride were used as washing agents, the former because it partly dissolved the granules; any supernatant adhering to the surface of granules should then be completely removed. Singly and doubly washed fractions had virtually the same dehydrogenase activities, whether saline or water were used for washing (Table 5). But for fumarase and aconitase, the opposite was found. Doubly washed granules had a higher fumarase activity than once washed granules, whether potassium chloride or water were used for washing. Aconitase activity was greatly increased by washing with water. Table 6 shows that all washings had both fumarase and aconitase activity. Thus, washing caused overall increases in the total activities of both enzymes. This is discussed further below.

Table 6. *Specific activities of washings from particulate fraction*

	Fumarase	Aconitase
First aqueous washing	38	20
Second aqueous washing	77	28
First saline washing	186	45
Second saline washing	Dry weights too low to measure but activities appreciable	

Table 7. *Coenzyme requirement of ethanol, malic, citric and glutamic dehydrogenases*

Dialysed S_2, from 10-sec. extract, was used. Values are means of three experiments.

	$Q_{M.B.}$		
Substrate	Without coenzyme	With 0·1 mg. DPN	With 0·1 mg. TPN
Ethanol	<1	59	11
L-Malate	10	23	15
Citrate	<0·5	1·0	3·5
L-Glutamate	<0·5	1·3	16

Table 8. *Effect of dialysis on the DPN requirement of malic and ethanol dehydrogenases*

Dialysed S_2 from various extracts was used.

		$Q_{M.B.}$	
Disintegration period (sec.)	Substrate	Without DPN	With 0·1 mg. DPN
10	L-Malate	2·7	10
30	L-Malate	8·3	15
90	L-Malate	8·0	15
90*	L-Malate	6·6	—
10	Ethanol	<0·5	34
30	Ethanol	<0·5	50

* Supernatant dialysed for 15 hr. and then for another 40 hr., each time against 60 vol. 0·9 % KCl.

Nucleotide-coenzyme requirement of the various dehydrogenases. The succinic and lactic dehydrogenases were independent of added coenzymes in all fractions tested.

The ethanol and malic enzymes were more active with DPN than with TPN (Table 7), whereas the citric and glutamic enzymes were more active with TPN than with DPN. All four enzymes, however, were activated by both coenzymes. Our assays showed that the DPN used for these experiments was free of TPN (Zwischenferment assay, Warburg, Christian & Griese, 1935); and that the TPN was free of DPN, there being no difference between chemical estimation of DPN+TPN (Colowick, Kaplan & Ciotti, 1951) and Zwischenferment assay. Whether TPN was enzymically converted into DPN in these preparations is not known, but this is possible according to the observations of von Euler & Adler (1938). The conversion of DPN into TPN is less likely since this process requires added adenosine triphosphate (Kornberg, 1950). Kornberg & Pricer (1951) have already reported two *iso*citric dehydrogenases in yeasts, specific for DPN and TPN respectively. The former also requires manganous chloride and adenosine 5-phosphate. In our extracts, these two substances were required for maximum activity in the presence of DPN, but the TPN-dependent enzyme seemed to predominate.

Dialysis of S_2 caused almost complete loss of ethanol, *iso*citric and glutamic dehydrogenase activities when these were tested without added coenzymes. However, only about 70 % or less of the total malic dehydrogenase activity was lost (Table 8). S_2, dialysed twice against a total of 120 vol. 0·9 % potassium chloride and tested without DPN, still retained almost half the activity shown in the presence of DPN. This suggests the occurrence of a DPN-independent malic dehydrogenase or firmer binding of DPN to this particular enzyme.

Evidence for the existence of bound coenzymes in the granules. In carefully prepared mitochondria from animal cells, dehydrogenases which in the soluble ('dissociated') form require pyridine nucleotide coenzymes, are fully active without added coenzymes. Green and associates have shown this to be due to bound coenzymes (Huenneckens & Green, 1950; Still & Kaplan, 1950). Yeast particles prepared from 10-sec. extracts also showed this property, but 30- or 90-sec. particles did not (Table 9).

The malic and ethanol dehydrogenases of 10-sec. particles were occasionally as active without added DPN as in the presence of DPN, but more often they showed only about half the full activity. The possibility of contamination by coenzymes from the supernatant was ruled out in two ways. One batch of 10-sec. particles was divided into three parts, one of which was washed as usual with the original

Distribution of enzymes in cell-free yeast extracts

By P. M. Nossal

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p. 66, col. 1, line 21:

Delete $Q_{M.B.}$ and bracket

Table 9. *Coenzyme dependence of granular ethanol and malic dehydrogenases*

W was centrifuged at 10000 *g* and the combined particles were washed as shown below.

			$Q_{M.B.}$			
			Ethanol		L-Malate	
Expt.	System	Notes	Without DPN	With 0·1 mg. DPN	Without DPN	With 0·1 mg. DPN
1	10-sec. particles	Washed as usual	41	46	15	24
		Washed with 2 vol. KCl	46	46	14	20
		Washed with 3 vol. KCl	33	37	12	17
2	10-sec. particles	Washed as usual	46	60	11	21
		Washed twice	38	46	9	19
		Washed thrice	19	26	9	19
3	30-sec. particles	Washed as usual	3	15	1	12
	90-sec. particles	Washed as usual	<0·5	4	<0·5	5

Table 10. *Fumarase and aconitase balance of fractionated extracts*

Rates of activity refer to 1 ml. of the original undiluted extract. Fractions all calculated on this basis. Values are means of the number of experiments cited.

			Rate of change of optical density (240 mμ.)/min./ml. × 1000							
					Particulate fractions		Washings of particulate fractions			
Disintegration period (sec.)	Activity measured	No. of experiments	Whole extract (1)	S_2 (2)	*A* (3)	*B* (4)	*A* (5)	*B* (6)	(2)+(3)+(4)+(5)+(6) (7)	% increase over original whole extract
10	Fumarase	3	1513	1420	129	204	46	29	1828	21
10	Fumarase	3	1020	950	←250*→		←80*→		1280	25
30	Fumarase	3	3227	3573	94	185	121	121	4094	27
90	Fumarase	2	4540	4620	16	26	216	171	5049	11
10	Aconitase	3	369	375	←37*→		←20*→		428	16
30	Aconitase	3	983	1100	20	41	39	32	1232	25
90	Aconitase	2	1540	1480	9	21	63	44	1617	5

* In these experiments, *W* was centrifuged at 10000 *g*, so that the sediment contains combined fractions *A* and *B*; these were washed together.

volume of 0·9 % potassium chloride, the others with 2 and 3 vol., respectively. The dehydrogenase activities of all three were almost as high without added DPN as with DPN. Secondly, three portions of another batch of 10-sec. particles were washed once (as usual), twice and three times, respectively, with the original volume of 0·9 % potassium chloride. Again, all three had high activities without added DPN. 30- and 90-sec. particles had only very low activities without added DPN.

10-sec. particles lost almost entirely these coenzyme-independent activities (but not so much their dehydrogenase activities with DPN) by storage at 1° for 24 hr., and also by 15-sec. shaking without Ballotini in our disintegrator. Both observations are in accordance with those for mitochondria. Carefully prepared yeast particles therefore seem to resemble mitochondria from animal cells in containing bound coenzymes.

Quantitative studies with fumarase and aconitase. In an attempt to ascertain the contribution of the particulate fraction to the total enzymic activities of the extracts, we have measured the fumarase and aconitase activities of unfractionated extracts and of all the individual fractions. Table 10 shows that in 10-sec. extracts the sum of the activities of individual fractions exceeded those of the original extract by 25 %. Similarly, when a whole 10-sec. extract was reconstituted from the individual fractions, its activities again exceeded those of the original extract. The difference between the original activities of unfractionated extracts and the sum of the activities of individual fractions was much smaller in 90-sec. extracts.

The specific activities of 90-sec. whole extracts were found to be up to twice as high as those of 10-sec. extracts made from the same batch of yeast (Table 11).

These findings suggest masked fumarase and aconitase activities in 10-sec. extracts. Masked activities seem to be associated with extracts containing enzymically active granules, and were therefore not found in 90-sec. extracts, in which the granules are inert. It was noted above (Table 5)

Table 11. *Effect of disintegration period on the fumarase and aconitase activities of whole extracts*

Concurrent 10-, 30- and 90-sec. disintegrations were carried out with 2 g. portions of the same yeast sample. Values are the means of three such experiments.

Disintegration period (sec.)	Amount used for test (mg. dry wt.)	Activity measured	Change in optical density/ min. × 1000	Specific activity
10	0·40	Fumarase	28	70
30	0·38	Fumarase	44	115
90	0·37	Fumarase	50	135
10	0·79	Aconitase	15	19
30	0·77	Aconitase	23	30
90	0·74	Aconitase	24	33

that double washing increased the activities of 10-sec. granules. We have also found that 24 hr. storage of 10-sec. particles in 0·9 % potassium chloride at 2° increased both fumarase and aconitase activities. Various explanations to account for this masking are discussed below.

DISCUSSION

This work has shown for the first time that granules rich in enzymes other than succinoxidase and cytochrome *c* reductase can be prepared from a micro-organism. Increasing periods of mechanical disintegration cause progressive losses of all enzymes, but especially the more soluble dehydrogenases and fumarase and aconitase, from granules to supernatant. It seems, then, that the intact cell contains granules rich in all these enzymes, and that our particulate fractions contain the microscopically visible granules which have been called mitochondria by many workers on purely histological grounds (Lindegren, 1949; Mudd *et al.* 1951; Sarachek & Townsend, 1953; Mundkur, 1953). The particulate fractions of 10-sec. yeast extracts also resemble the mitochondria prepared from animal tissues in containing bound coenzymes. On the evidence presented here, it seems that yeast cells do not contain different types of granules rich in respiratory enzymes, such as the mitochondria and microsomes of animal cells. The similarity in enzymic content of particulate fractions *A* and *B* argues against such a distinction, as does the finding that increasing periods of mechanical disintegration apparently cause conversion of the more readily sedimentable fraction *A* into the less readily sedimentable fraction *B*.

The discrepancies between our results and those of Hirsch (1952) and Slonimsky & Hirsch (1952) can probably be explained by the differences in disintegration procedure. Whereas our best extracts were prepared by 10-sec. disintegration, the equivalent period on the Towers Shaker must have been of the order of 60 min. During that period, most of the more soluble dehydrogenases would be removed from the granules. Therefore, the granules in Hirsch and Slonimsky's extracts resemble our 90-sec. granules in being deficient in the more soluble enzymes and also in bound coenzymes.

The more quantitative results with fumarase and aconitase give some indication of the contribution of granules to the total enzyme content of the cell. In 10-sec. extracts, the particulate fraction accounts for about 15 % of the dry weight, and the specific fumarase activity of the particles is up to twice that of the supernatant. The particulate fumarase activity is reduced threefold in 30-sec. extracts. It is reasonable to assume, therefore, that in the intact cell most of the fumarase activity is associated with granules. This may well apply also to the soluble dehydrogenases, and further strengthens the argument that yeast cells contain granules akin to the mitochondria of animal and plant cells.

Several sets of findings have indicated masked fumarase and aconitase activities in the extracts, and the masking is associated with the granular fraction. No natural inhibitors of fumarase and aconitase are known. It seems unlikely that the masked activities recorded are due to partial inhibition. Two other explanations cover the findings. The first is partial structural separation of enzymes and their substrates in carefully prepared granules; this could be due to a membrane such as that described for plant mitochondria by Farrant, Robertson & Wilkins (1953) or to orientation of the organized enzymes. Washing, especially with water, and storage could lead to deterioration of the granules and thus to increased activity. Increasing disintegration periods cause increasing mechanical disruption of particulate fractions, again liberating granular enzymes.

The second explanation is metabolic 'removal' of the products of fumarase and aconitase action, possibly by reduction. On the other hand, no enzymes of this type have been found to occur in yeast cells in activities even approaching those of fumarase.

We hope to carry out electron-microscopic studies of granular structure to examine further the first theory.

The high malic, glutamic, succinic and *iso*citric dehydrogenase, fumarase and aconitase activities of these extracts again suggest participation of the Krebs's cycle in yeast respiration. However, until actively respiring, cell-free extracts can be prepared, the main mechanism of the carbohydrate and alcohol oxidation remains controversial (see Weinhouse & Millington, 1947; Martius & Lynen, 1950; Foulkes, 1951 on one hand; and Krebs, 1949; Krebs *et al.* 1952 on the other).

SUMMARY

1. Cell-free yeast extracts were prepared by 10-, 30- and 90-sec. high-speed mechanical disintegration, and residual intact cells removed by centrifugation at 1000 *g*. The extracts were then fractionated into particulate fraction *A* (sedimented at 3500 *g*), particulate fraction *B* (sedimented at 10 000 *g*) and non-sedimentable supernatant.

2. The following substrates, in decreasing order of activity, caused methylene-blue reduction in Thunberg tubes with whole unfractionated extracts: ethanol, L-malate, *iso*citrate, lactate, L-glutamate, citrate and succinate. Fumarase was about three times as active as aconitase.

3. Increasing periods of mechanical disintegration cause a progressive migration of all enzymes tested from particles to supernatant.

4. The enzyme content of both particulate fractions is so similar that they are probably not separate entities.

5. Malic and ethanol dehydrogenase activities are maximal with DPN, 'citric' and glutamic dehydrogenase activities with TPN. All four enzymes are activated to some extent by both coenzymes. The other enzymes require no added coenzymes.

6. The particles of 10-sec. extracts are rich in all enzymes examined and contain bound coenzymes which are not removed by repeated washing, but are lost by 24 hr. storage at 2° or by mechanical treatment. The particles of 30- and 90-sec. extracts contain less, if any, bound coenzymes.

7. Several sets of findings point to masked fumarase and aconitase activities in unfractionated extracts, and the masking is associated with the particulate fraction. Various possible explanations are discussed.

8. It is suggested that in the intact cell the microscopically visible granules, which some authors have called mitochondria on histological grounds, are active sites of enzyme activities concerned with respiration.

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A Note on the Free Amino Acid Content of Rat Brain

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Some twenty different amino acids have been identified by paper chromatography in the free amino-nitrogen fraction of the brain (Roberts, Frankel & Harman, 1950; Astrup, Carlström & Stage, 1951; Walker, 1952). The quantitative values obtained by different investigators for the glutamine and glutamic acid contents show fairly good agreement (Krebs, Eggleston & Hems, 1949; Waelsch, 1949; Dawson, 1950): but few reliable figures are available for the concentrations of the other amino acids in fresh brain tissue. Awapara, Landua & Fuerst (1950) estimated the concentrations of four amino acids in the rat brain by extracting the ninhydrin-reacting spots from paper chromatograms. A number of free amino acids in rat brain have also been estimated by a microbiological method (Schurr, Thompson, Henderson, Williams & Elvehjem, 1950) and the values obtained were found to be affected by procedures such as exercising or cooling the animals before they were killed (Williams, Schurr & Elvehjem, 1950). Their control values were obtained on anaesthetized animals; but it appeared doubtful whether these values should be accepted as normal, since Dawson (1951) has found the glutamic acid level of the brain to be affected by thiopentone anaesthesia.

The present study started with the observation that there is a fairly rapid post-mortem liberation of amino acids in brain tissue incubated at a neutral pH, owing to the action of a proteinase that is active under these conditions (Ansell & Richter, 1954). It appeared that post-mortem changes of this kind might be a serious source of error in estimations of the amino acids of brain tissue; the pattern of amino-nitrogen distribution in the rat brain was therefore re-investigated in brain tissue rapidly frozen in liquid oxygen to minimize any post-mortem change. The possible effects of the method of killing and of anaesthesia on the brain amino acid content were investigated at the same time. The amino acids were estimated by the colorimetric method of Roberts & Frankel (1950) after a preliminary chromatographic separation.

EXPERIMENTAL

Young rats of about 50 g. (Wistar albino strain) were used throughout. Anaesthesia was induced by intraperitoneal injection of thiopentone ('Pentothal'; 60 mg./kg.), and the temperature of the anaesthetized animals was maintained by raising the environmental temperature and checking with a rectal thermometer. The rats were decapitated by a single sharp blow with a hammer on a razor placed above the neck and the heads frozen immediately by dropping into an insulated beaker containing liquid oxygen. Rats were also killed, where so stated, by dropping them whole into liquid oxygen. The frozen brains were dissected out without allowing them to thaw, crushed in a steel crusher, and the powder was dropped into a weighed centrifuge tube containing 5 ml. 12% (w/v) trichloroacetic acid at 0°. The tube was vigorously shaken, weighed and centrifuged. The total volume was taken as 5 ml. +0·8 ml. for each g. of brain. The solution was heated at 70° for 75 min. to convert glutamine into pyrrolidonecarboxylic acid and ammonia, and then stored at 4°.

Determination of total amino nitrogen. The total amino nitrogen was estimated by the colorimetric method of Moore & Stein (1948), the extinction coefficient being measured at 570 mμ.; glutamic acid was used as a standard. An ammonia standard was also used so that the total colour yield from the extract could be corrected for that due to ammonia, which was determined independently by the method of Conway (1939).

Paper chromatography. The tissue extracts were extracted twice with an equal volume of ether, which removed 95% of the trichloroacetic acid. They were then desalted by the method of Consden, Gordon & Martin (1947), care being taken to keep the extracts cool. The volume of extract used corresponded to 20–50 mg. fresh brain tissue, and the ninhydrin-reacting spots on two-dimensional phenol : collidine chromatograms (Dent, 1948) were identified by markers, by their stability to acid hydrolysis and by their reaction with $CuCO_3$ (Crumpler & Dent, 1949).

The quantitative estimation of the ninhydrin-reacting substances was carried out by the method of Roberts & Frankel (1950), after preliminary trials in which consistent results were obtained. Extinction coefficients were measured with an Ilford filter no. 812 (570 mμ.) because amino acids yield a colour having minimum transmission at this wavelength (Bull, Hahn & Baptist, 1949). It was found that although the Beer-Lambert law was obeyed over the range studied, the colour yield/μmole varied for individual amino compounds; there were also slight day-to-day variations. In each series of estimations control chromatograms of known amounts of amino compounds were therefore run concurrently and the amounts calculated by reference to the standards prepared at the same time.

RESULTS

The effect of the method of killing. In a series of preliminary experiments free amino nitrogen and amide nitrogen + ammonia (approximately equivalent to the glutamine) were determined in groups of

rats that had been killed by various different procedures. There is evidence that violent stimulation, such as is caused by decapitation, may affect the free ammonia and other metabolites in the brain (Richter & Dawson, 1948). The amino-nitrogen concentration did not appear to be greatly affected by the method of killing and no significant differences were observed, provided the tissue was frozen without delay (Table 1): but slightly lower amino-nitrogen values were noted in two groups of animals that had been anaesthetized before killing. Further experiments were therefore carried out to see if this difference was attributable to the anaesthesia or due to chance.

The effect of anaesthesia. Dawson (1951, 1953) reported that thiopentone anaesthesia reduces the glutamic acid level in the rat brain; but Williams *et al.* (1950) failed to observe any effect of anaesthesia on a number of other amino acids which they estimated in the brain. The question appeared to be worth pursuing, since some of the 'normal' figures for the brain amino acids given in the literature were obtained on anaesthetized animals and an effect of anaesthetics on the nitrogen metabolism is not without interest. In the present study the total amino-nitrogen content of the brain in rats anaesthetized for various periods of time with thiopentone was compared with figures obtained under similar conditions for a series of unanaesthetized litter-mate controls (Table 2). Care was taken to maintain the temperature of the anaesthetized animals so that the results would be strictly comparable. The results showed a significantly lower amino-nitrogen content in the anaesthetized group ($P < 0.05$). However, the difference was small, amounting to only 2·5 mg. amino N/100 g. fresh brain. This was of the same order as the fall in the glutamic acid level in anaesthesia (about 2–3 mg. amino N/100 g. brain) observed by Dawson. It would appear from these figures that the effect is mainly or wholly accounted for by a fall in the glutamic acid level.

Table 1. *The effect of the method of killing on free amino nitrogen and (amide nitrogen + ammonia nitrogen) in rat brain*

The figures give mean values for duplicate determinations on the number of animals indicated. The anaesthetized animals received 60 mg. thiopentone/kg. body wt.

No. of rats	Method of killing	Amino N (mg./100 g. brain)	S.E.M.	Amide N + ammonia N (mg./100 g. brain)	S.E.M.
3	Decapitated and head frozen immediately	42·2	±1·57	7·5	±0·49
4	Animal frozen whole	43·4	±1·58	9·4	±0·38
7	Anaesthetized and frozen whole	41·2	±1·55	7·4	±0·51
6	Anaesthetized, decapitated and head frozen immediately	39·8	±1·17	9·8	±0·45

Table 2. *The effect of anaesthesia on free amino nitrogen and (amide nitrogen + ammonia nitrogen) in rat brain*

The animals were killed by decapitation and the heads immediately frozen in liquid oxygen. The anaesthetized animals received 60 mg. thiopentone/kg. body wt. The figures give the means of duplicate determinations.

		mg./100 g. fresh brain			
		Unanaesthetized litter-mate controls		Anaesthetized	
Rat no.	Duration of anaesthesia (min.)	Amino N	(Amide N + ammonia N)	Amino N	(Amide N + ammonia N)
1–2	72	43·8	8·5	44·9	10·0
3–4	77	41·4	8·5	38·6	9·6
5–6	104	41·0	9·0	40·9	10·8
7–8	77	45·7	9·1	35·3	8·8
9–10	95	37·3	8·4	38·3	9·1
11–12	45	42·2	8·8	40·7	10·5
13–14	55	44·5	7·7	41·2	10·7
15–16	60	48·2	8·0	41·0	8·8
17–18	60	44·0	8·5	41·0	8·6
19–20	57	43·3	8·8	42·5	8·8
21–22	60	38·3	7·8	37·8	8·4
	Mean	42·7	8·5	40·2	9·5
	S.E.M.	±0·90	±0·14	±0·75	±0·26

Table 3. *Concentrations* (mg. *amino* N/100 g. *fresh brain*) *of amino acids and related compounds in rat brain*

The animals included in brackets were litter mates. The figures for total amino N exclude glutamine.

Rat no.	Glutamic acid	Aspartic acid	Glutathione*	*O*-Phosphoryl-ethanolamine	Glycine	Serine	Taurine	Alanine	γ-Amino-butyric acid	Total amino N
1	11·8	4·1	—	2·3	1·6	1·5	5·4	0·9	3·1	41·7
2	12·0	4·5	—	2·2	2·2	1·5	6·4	1·3	3·8	45·9
3	14·1	3·9	2·4	1·9	1·5	1·4	8·8	0·6	2·8	44·4
4	14·6	3·3	2·5	1·7	1·7	1·6	7·8	0·7	2·3	45·2
5	13·7	3·3	2·4	2·7	1·9	(0·7)	9·3	0·4	2·2	43·4
Mean	13·2	3·8	2·4	2·2	1·8	1·5	7·5	0·8	2·8	44·1
Other workers	12·9† 15·0§	5·1†	—	1·6†	—	—	3·3†	—	1·6‡	—

* Observed only on chromatograms treated with hydrogen peroxide and ammonium molybdate (Dent, 1948).
† Awapara, Landua & Fuerst (1950).
‡ Awapara, Landua, Fuerst & Seale (1950).
§ Dawson (1950).

The figures also showed a small but significant rise in the amide-nitrogen (glutamine) fraction in anaesthesia ($P < 0{\cdot}01$) (cf. Dawson, 1951). The effects of thiopentone anaesthesia on the amino nitrogen and amide nitrogen were confirmed qualitatively by the visual comparison of four pairs of chromatograms prepared from desalted brain extracts of normal and anaesthetized rats. A careful comparison showed in every case a slight fall in the intensity of the glutamic acid spot and an apparent slight increase in the intensity of the spots for glutamine and γ-aminobutyric acid. The intensity of the other spots remained unchanged as far as could be judged by this method; but Dawson (1953) has recently shown that the aspartic acid content is increased in thiopentone anaesthesia. The present results therefore confirm Dawson's finding of a fall in the glutamic acid level and a rise in the glutamine in anaesthesia; they also agree with the implication of Williams *et al.* (1950) that certain other amino acids are not significantly affected.

The concentration of individual amino acids in brain tissue. The values obtained for the concentration of nine amino acids and related compounds, and for the total amino nitrogen in fresh rat brain are given in Table 3. Each figure represents the mean of two determinations on the same trichloroacetic acid extract prepared from frozen whole brain tissue. The rats were killed by decapitation and the heads immediately frozen in liquid oxygen.

Glutamine was converted into pyrrolidonecarboxylic acid and was not determined individually or included in the figure for the total amino nitrogen. If the glutamine amino-nitrogen content of rat brain is taken as 7 mg./100 g. tissue, then the total free amino nitrogen is 49·7 mg./100 g. (using the mean of the 'normal' values in Table 2). This figure agrees approximately with the figures of 52 mg. reported by Awapara, Landua & Fuerst (1950) and 50·5 mg. which can be calculated from the results of Dawson (1950). It may be seen that the three related compounds glutamine, glutamic acid and γ-aminobutyric acid accounted for 45 % of the total amino nitrogen.

The figures for the individual amino compounds are of the same order as those reported by Awapara, Landua & Fuerst (1950) and Awapara, Landua, Fuerst & Seale (1950) for the five compounds estimated by them, except for taurine which was found at about double the concentration they gave. However, the present figure obtained for taurine would agree with the qualitative observations of Roberts *et al.* (1950) and of Boulanger & Biserte (1951), who reported that it was present in relatively high concentration. The spots due to threonine and cysteic acid (derived from cyst(e)ine and observed on chromatograms treated with hydrogen peroxide and ammonium molybdate (Dent, 1948)) were too faint for quantitative estimation by the present methods and their intensification by using a larger quantity of extract was prohibited, since it would have led to errors due to the overlapping with other spots.

DISCUSSION

The amino acids of the brain may be regarded as falling into two main groups. There is the large group of amino acids present in relatively low concentration corresponding to the amino acid 'pool' in other tissues: these are presumably concerned in the processes of synthesis and breakdown of the tissue proteins, which are constantly taking place. There is, secondly, the small group of related compounds, glutamine, glutamic acid and γ-aminobutyric acid, which are present in relatively high concentrations and to which a special function must be ascribed. Glutamic acid is unique among the amino acids of the brain in its ability to undergo oxidation, amidation, transamination and decarboxylation, and it appears to occupy a central position in the metabolism of the brain.

The nine amino compounds estimated individually in the present study corresponded altogether to 36 mg. amino N/100 g. brain. If 7 mg./100 g. brain is taken for the glutamine amino nitrogen and 3 mg./100 g. brain for the group of twelve less abundant amino acids (including threonine) estimated microbiologically by Schurr, Thompson, Henderson, Williams & Elvehjem (1950), this adds up to 46 mg./100 g. brain, which accounts for 92·5% of the total amino nitrogen. In the remaining 7·5% will occur a number of other amino compounds present in too small a concentration to give satisfactory spots under these conditions on chromatograms.

Roberts *et al.* (1950) reported an acid-labile spot X appearing 'underneath' glutamic acid (also designated as 'O' in a later paper by Roberts & Frankel (1950)) on phenol : collidine chromatograms of ethanolic extracts of mouse brain: a spot 'under glutamic acid' was also mentioned by Awapara, Landua & Fuerst (1950). This unidentified spot was presumably *O*-phosphorylethanolamine, which has now been identified in rat brain extracts (Ansell & Dawson, 1951).

Roberts *et al.* (1950) also reported a further substance 'X' in mouse brain extracts which disappeared on acid hydrolysis, (designated 'P' in a later paper by Roberts & Frankel, 1950). This substance scarcely moved in phenol and 'collidine' runs and in this respect resembles hydroxylysine phosphate, which was identified in calf embryo muscle by Gordon (1949) and reported by Astrup *et al.* (1951) to be present in ox brain extracts. A spot in this position was never observed on chromatograms of desalted extracts prepared from rat brain frozen in liquid oxygen, though a ninhydrin-reacting streak was sometimes seen on chromatograms of undesalted extracts. Astrup *et al.* do not state how soon after death their extracts were prepared: but they used for their chromatograms an amount of extract equivalent to about 10 times the amount of brain tissue used in the present study, and it is therefore very likely that such a spot would not be seen under our conditions. These workers also reported the presence of a small amount of β-alanine and carnosine in ox brain extracts.

The only peptide estimated in the present study was glutathione, but other peptides are also present in rat brain tissue, in an amount corresponding to about 20 mg. peptide-bond nitrogen/100 g. of brain (Ansell & Richter, 1954). The results of Schurr, Thompson, Henderson & Elvehjem (1950) and of Boulanger & Biserte (1951) indicate that peptides of leucine, isoleucine, valine, histidine, proline, alanine, glycine, aspartic acid and γ-aminobutyric acid are present in brain tissue.

SUMMARY

1. The free amino-nitrogen content of rat brain falls by about 2·6 mg./100 g. tissue in thiopentone ('Pentothal') anaesthesia. This is attributable mainly to a fall in the glutamic acid level.

2. Nine amino compounds visible on paper chromatograms were estimated in deproteinized extracts of rat brain which had been frozen in liquid oxygen immediately after death.

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Studies in Detoxication

59. THE METABOLISM OF ALKYLBENZENES. THE BIOLOGICAL REDUCTION OF KETONES DERIVED FROM ALKYLBENZENES

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Recently we showed that acetophenone was asymmetrically reduced in rabbits to (−)-methylphenylcarbinol (Smith, Smithies & Williams, 1954). Five more ketones, namely, ethyl phenyl, phenyl propyl, benzyl methyl, benzyl ethyl, and methyl phenethyl ketones have now been studied, and it appears that some of these are also asymmetrically reduced in the rabbit. The glucuronides (glucosiduronic acids) isolated in this work were needed as reference compounds for other work in progress on alkylbenzenes.

EXPERIMENTAL

Melting points are corrected and optical rotations are for 1% solutions in chloroform, the error being ±1°.

Isolation of glucuronides

From ethyl phenyl ketone. Propiophenone (b.p. 218°; 2·5 g.) was fed to each of four rabbits. The urine was collected for 24 hr. and the glucuronide gum (10·3 g.) isolated through its basic lead salt. This gum was methylated and acetylated (cf. Kamil, Smith & Williams, 1951) and the crude triacetyl methyl ester (9·2 g.) had $[\alpha]_D$ −72°. Recrystallization from methylated spirit yielded 4·91 g. of white needles with $[\alpha]_D^{25}$ −80°. The rotation remained unchanged on further crystallization and the pure *methyl*((−)-*ethylphenylcarbinyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate* had m.p. 127° and $[\alpha]_D^{20}$ −80°. (Found: C, 58·5; H, 6·3. $C_{22}H_{28}O_{10}$ requires C, 58·3; H, 6·2%.) Examination of the mother liquors from this ester did not reveal the presence of the (+)-isomer.

From phenyl n-*propyl ketone.* Butyrophenone (b.p. 232°; 2 g.) was fed to each of six rabbits and from the 24 hr. urine 12·2 g. of glucuronide gum were isolated. Part of this (7 g.) was converted into a gummy triacetyl methyl ester (5·75 g.; $[\alpha]_D^{20}$ −63°). This ester was eventually crystallized with great difficulty from 50% (v/v) aqueous acetic acid by allowing the solution to evaporate slowly. *Methyl* ((−)-*phenyl*-n-*propylcarbinyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate* (1·84 g.) was obtained as colourless needles, m.p. 104° (from aqueous ethanol) and $[\alpha]_D^{20}$ −78·8°. (Found: C, 59·0; H, 6·7. $C_{23}H_{30}O_{10}$ requires C, 59·2; H, 6·5%.) Further examination of the mother liquors from this ester failed to reveal the presence of the (+)-isomer.

From benzyl methyl ketone. The ketone (b.p. 214°; 2·25 g.) was fed to each of four rabbits and from the 24 hr. urine, 12 g. of glucuronide gum were isolated. This yielded 10·3 g. of crude triacetyl methyl ester with $[\alpha]_D$ −21·2°. Purification of this ester from ethanol and *iso*propyl ether eventually yielded 4·76 g. of pure *methyl* ((+)-*benzylmethylcarbinyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate*, as needles m.p. 118° and $[\alpha]_D^{20}$ −24°. (Found: C, 58·35; H, 6·4. $C_{22}H_{28}O_{10}$ requires C, 58·35; H, 6·2%.) The mother liquors from this ester did not appear to contain any of the (−)-isomer.

From (±)-*benzylmethylcarbinol.* This carbinol, b.p. 105°/20 mm., was prepared in 80% yield by reducing the ketone with lithium aluminium hydride (Brown, 1951). Quantitative estimation of the glucuronic acid output after feeding the carbinol (0·5 g./kg.) to three animals showed that about 43% was excreted as conjugated glucuronic acid by rabbits. There was also a small rise (corresponding to about 3% of the dose) in excretion of ethereal sulphates, suggesting a slight nuclear oxidation. The glucuronides of both isomers of benzylmethylcarbinol were isolated after feeding the carbinol.

The (±)-carbinol was fed to six rabbits (2·5 g. each). The gummy triacetyl methyl ester was prepared in the usual manner and repeated recrystallization from 50% aqueous ethanol yielded methyl ((+)-benzylmethylcarbinyl tri-*O*-acetyl-β-D-glucosid)uronate (2·4 g.), m.p. 120° and $[\alpha]_D^{20}$ −24°. (Found: C, 58·7; H, 6·5%.) This ester did not depress the m.p. of the glucuronide obtained by feeding benzyl methyl ketone (see above).

The (−)-isomer was obtained in another experiment. (±)-Benzylmethylcarbinol was fed to seven rabbits (2 g. each). From the 24 hr. urine 14·2 g. of glucuronide gum was obtained and this yielded 11 g. of crude triacetyl methyl ester with $[\alpha]_D^{25}$ −30°. Three recrystallizations from ethanol yielded material with decreased rotation ($[\alpha]_D$ −26°), and from the combined mother liquors after evaporation, 2·2 g. of material with $[\alpha]_D$ −34° was obtained. Four recrystallizations of the latter material from *iso*propyl ether gave 0·67 g. of crystals with $[\alpha]_D^{25}$ −46° and m.p. 116°. Further recrystallizations from 80% ethanol or *iso*propyl ether raised the m.p. but the rotation remained constant. Finally, the *methyl* ((−)-*benzylmethylcarbinyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate* (0·35 g.) was obtained as colourless needles, m.p. 127·5° and $[\alpha]_D^{25}$ −46°. (Found: C, 58·3; H, 6·2. $C_{22}H_{28}O_{10}$ requires C, 58·35; H, 6·2%.)

From methyl phenethyl ketone. The ketone (b.p. 234°), prepared as described by Vogel (1948), was fed to four rabbits (2 g. each) and the urine (500 ml.) collected during 24 hr. The urine gave a strong naphthoresorcinol reaction and slightly reduced Benedict's reagent but not Fehling's solution. It gave a positive iodoform test for methyl ketone and methyl carbinol and a slight positive test with Brady's reagent for ketones. It yielded 5·5 g. of glucuronide gum and 4·8 g. of crude amorphous triacetyl methyl ester with $[\alpha]_D$ −33°. This ester crystallized with difficulty from

aqueous methanol yielding 0·85 g. of semi-crystalline solid with $[\alpha]_D$ – 26·5°. Evaporation of the mother liquors yielded a gum with $[\alpha]_D$ – 37° which resisted all attempts at crystallization. The solid material on recrystallization from aqueous methanol yielded 0·1 g. of colourless needles melting sharply at 120° and $[\alpha]_D^{20}$ – 27°. (Found: C, 59·1; H, 6·6. $C_{23}H_{30}O_{10}$ requires C, 59·2; H, 6·5%.) This is probably *methyl*((+)-*methylphenethylcarbinyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate*.

From benzyl ethyl ketone. The ketone (10 g., b.p. 225°), prepared according to Blatt (1943), was fed to four rabbits and the urine collected for 48 hr. The naphthoresorcinol reaction was positive, but Fehling's, Benedict's and Brady's reagents gave negative tests. The urine yielded 7·2 g. of glucuronide gum and 5·6 g. of gummy triacetyl methyl ester with $[\alpha]_D^{23}$ – 27·5°. A solution of the ester in ethanol slowly deposited crystals (0·16 g. with $[\alpha]_D^{23}$ – 19·5° and a further crop of 0·13 g. with $[\alpha]_D$ – 22°). Recrystallization of the first crop from *iso*propyl ether gave 90 mg. of fine needles, m.p. 113° and $[\alpha]_D^{23}$ – 22°. (Found: C, 59·15; H, 6·7. $C_{23}H_{30}O_{10}$ requires C, 59·2; H, 6·5%.) This compound is probably *methyl*((+)-*benzylethylcarbinyl tri*-O-*acetyl*-β-D-*glucosid*)*uronate*. The residual gum from these crystals had a higher negative rotation than this ester, and had not crystallized after several months.

DISCUSSION

Thierfelder & Daiber (1923) fed a number of mixed ketones to rabbits and isolated the glucuronides formed as metallic salts. The molecular rotations of these compounds are quoted in Table 1. These workers made no specific comment on the stereochemistry of the glucuronides. Recently, Smith *et al.* (1954) have shown that acetophenone is converted in rabbits into the glucuronide of (−)-methylphenylcarbinol. An examination of the rotations of the glucuronides given in Table 1 suggests that propiophenone and butyrophenone, like acetophenone, are converted in rabbits into the glucuronides of the corresponding (−)-carbinols. Benzyl *iso*propyl ketone, however, produced a glucuronide whose rotation was much less than the other three and it is possible that in this case a (+)-carbinol is produced (cf. benzyl methyl ketone, below).

In the present work the glucuronides obtained after feeding the ketones have been characterized as triacetyl methyl esters, whose rotations are quoted in Table 2, from which it appears that propio- and butyro-phenones are reduced in rabbits to carbinols of the same sign of rotation as (−)-methylphenylcarbinol, since the molecular rotations of the triacetyl methyl esters of the glucuronides are almost identical. (−)-Ethylphenylcarbinol and (−)-phenyl-*n*-propylcarbinol on rotation arguments have the same relative configuration as (−)-methylphenylcarbinol (Freudenberg, 1932). It thus appears that aceto-, propio- and butyrophenones are asymmetrically reduced in rabbits to carbinols configuratively related to L-(−)-glyceraldehyde.

With benzyl methyl ketone, however, the triacetyl methyl ester of the glucuronide has a low

Table 1. *Glucuronides isolated by Thierfelder & Daiber* (1923) *after feeding certain ketones to rabbits*

Ketone fed	Formula of glucuronide	$[\alpha]_D$ in water (°)	$[M]_D$ (°)
$Ph.CO.CH_3$	$C_{16}H_{17}O_7K$, $1·5H_2O$	−124	−450
$Ph.CO.CH_2.CH_3$	$C_{15}H_{19}O_7K$	−126	−441
$Ph.CO.CH_2.CH_2.CH_3$	$(C_{16}H_{21}O_7)_2Cd$	−109	−415
$Ph.CH_2.CO.CH(CH_3)_2$	$C_{17}H_{23}O_7K$	−55	−208

Table 2. *Optical rotations of the triacetyl methyl esters of glucuronides isolated after feeding certain ketones to rabbits*

Compound fed	Carbinol present in glucuronide	Rotation (*c*, 1 in $CHCl_3$) of triacetyl methyl ester of glucuronide of carbinol: $[\alpha]_D$ (°)	$[M]_D$ (°)
$Ph.CO.CH_3$	(−)-$Ph.CHOH.CH_3$	−83*	−364
(±)-$Ph.CHOH.CH_3$	(−)-$Ph.CHOH.CH_3$	−83*	−364
	(+)-$Ph.CHOH.CH_3$	−15*	−66
$Ph.CO.CH_2.CH_3$	(−)-Ph $CHOH.CH_2.CH_3$	−80	−362
$Ph.CO.CH_2.CH_2.CH_3$	(−)-$Ph.CHOH.CH_2.CH_2.CH_3$	−79	−368
$Ph.CH_2.CO.CH_3$	(+)-$Ph.CH_2.CHOH.CH_3$	−24	−108
(±)-$Ph.CH_2.CHOH.CH_3$	(+)-$Ph.CH_2.CHOH.CH_3$	−24	−108
	(−)-$Ph.CH_2.CHOH.CH_3$	−46	−208
$Ph.CH_2.CO.CH_2.CH_3$	(+)-$Ph.CH_2.CHOH.CH_2.CH_3$†	−22	−103
$Ph.CH_2.CH_2.CO.CH_3$	(+)-$Ph.CH_2.CH_2.CHOH.CH_3$†	−27	−126

* From Smith *et al.* (1954). † See text.

$[\alpha]_D$ of $-24°$. This glucuronide could be derived from (+) or (−)-benzylmethylcarbinol. (±)-Benzylmethylcarbinol was therefore fed, and the expected two diastereoisomeric glucuronides were isolated as triacetyl methyl esters, one with $[\alpha]_D - 24°$ identical with the compound isolated after feeding the ketone, and the other with $[\alpha]_D - 46°$. The glucuronide with the higher negative rotation was obviously derived from (−)-benzylmethylcarbinol and the other from the (+)-isomer. Thus it was clear that benzyl methyl ketone was reduced in the rabbit to (+)-benzylmethylcarbinol. (+)-Benzylmethylcarbinol has been correlated chemically with L-(−)-glyceraldehyde (Levene & Walti, 1931; see also Freudenberg, 1932). Thus all the four ketones considered so far appear to be asymmetrically reduced in the rabbit to secondary carbinols related to L-(−)-glyceraldehyde.

With the other two ketones, methyl phenethyl ketone and benzyl ethyl ketone, similar conclusions cannot be drawn, since the yields of crystalline triacetyl methyl ester isolated were very small compared with the amount of non-crystalline ester actually obtained. Furthermore, the rotation of the crystalline ester was less negative than that of the bulk of material from which it was isolated although the differences were not very great. Thus with methyl phenethyl ketone, 0·85 g. of crystalline ester with $[\alpha]_D - 26·5°$ was separated from 4·8 g. of non-crystalline ester with $[\alpha]_D - 33°$, whereas with benzyl ethyl ketone, 0·16 g. of crystalline ester, $[\alpha]_D - 19·5°$ came from 5·6 g. of non-crystalline ester, $[\alpha]_D - 27·5°$. The non-crystalline esters could have contained the other diastereoisomer but this could not be proved since they failed to crystallize. (Other metabolites of these ketones are probably present and complicate matters.) Methyl phenethyl ketone is reported to yield phenaceturic acid in the dog (Dakin, 1908–9; Hermanns, 1913). Suggestions as to the stereochemical nature of these crystalline esters, however, can be made. The crystalline methyl (methylphenethylcarbinyl triacetyl glucosid)uronate, $[M]_D - 126°$, and methyl (benzylethylcarbinyl triacetyl glucosid)uronate, $[M]_D - 103°$, have rotations very similar to that of methyl ((+)-benzylmethylcarbinyl triacetyl glucosid)uronate, $[M]_D - 108°$. The $[M]_D$ of the (−)-form of the last ester is $-208°$. Furthermore, according to Freudenberg (1932), the corresponding optical forms of the configuratively related carbinols

$Ph.CH_2.CHOH.CH_3$ ($[M]_D$ 38·5°),

$Ph.CH_2.CHOH.C_2H_5$ ($[M]_D$ 51·5°)

and $Ph.CH_2.CH_2.CHOH.C_2H_5$ ($[M]_D$ 31°)

have the same sign of rotation, the (+)-forms being related to (−)-methylphenylcarbinol ($Ph.CHOH.CH_3$). On these grounds it is suggested that in methyl (methylphenethylcarbinyl triacetyl glucosid)uronate, m.p. 120°, and methyl (benzylethylcarbinyl triacetyl glucosid)uronate, m.p. 113°, the aglycones are in the (+)-form.

SUMMARY

1. Ethyl phenyl, phenyl *n*-propyl, benzyl methyl, benzyl ethyl and methyl phenethyl ketones are partly reduced in the rabbit to secondary carbinols which are excreted conjugated with glucuronic acid. The glucuronides have been isolated and characterized as triacetyl methyl esters.

2. Ethyl phenyl and phenyl *n*-propyl ketones appear to be reduced to the corresponding (−)-carbinols and benzyl methyl ketone to the (+)-carbinol. These carbinols are configuratively related to (−)-methylphenylcarbinol.

3. (±)-Benzylmethylcarbinol gives rise in rabbits to the glucuronides of both isomers of the carbinol.

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The Determination of the Optical Configuration of Naturally Occurring Amino Acids using Specific Enzymes and Paper Chromatography

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The problem of determining the optical configuration of naturally occurring amino acids has excited increasing attention of late. It has been known for some time as the result of direct isolation methods that the amino acids in the common vegetable and animal proteins are all of the same L configuration. The existence of D forms, however, has been suspected (Kögl & Erxleben, 1939) and denied (Chibnall, Rees, Williams & Boyland, 1940) in cancer tissues, and has been proved in the case of certain antibiotics of bacterial origin (see reviews in *Biochem. Soc. Symposia*, no. 1, 1948). There is little knowledge, however, of the configuration of the amino acids occurring in the free form in tissue fluids, although in discussions as to the significance of these compounds, the tacit assumption is usually made that they are in fact all of L configuration and are therefore immediately available for protein synthesis. Nevertheless, the fact that there exist in certain tissues, such as sheep's kidney cortex, large quantities of enzyme specifically capable of oxidizing D-amino acids, suggests that these latter compounds may occur at some stage of metabolism. More recently (Auclair & Patton, 1950) good evidence of the presence of D-alanine in insect blood has been obtained.

It is clear that any method of analysis, to be of practical value, must be capable of application to crude biological fluids of largely unknown composition and which may contain only μg. quantities of the amino acid in question. The only feasible tool is therefore an enzyme able specifically to act upon amino acids of one or other configuration, with paper chromatographic analysis of the mixture as a test of reaction or otherwise.

D- and L-amino acid oxidases come to mind as the most readily available enzymes for such a purpose. Lipmann, Behrens, Kabat & Burk (1940) and Lipmann, Hotchkiss & Dubos (1941) have already used the D oxidase in an attempt to determine the configuration of the amino acids in hydrolysates of various proteins. They performed the reactions in Warburg flasks and by following the oxygen uptake and ammonia production drew conclusions as to whether D- or L-amino acids were present and in what percentage of the total amino nitrogen. The method attained greater specificity when the enzyme solution of D-amino acid oxidase was sprayed directly on to the individual amino acids separated from each other on paper chromatograms. Synge (1949), for instance, was able to show in this way that gramicidin contained D-leucine and D-valine, while Jones (1948) likewise identified D-leucine and L-threonine in aerosporin. Both these workers, however, dealt with mixtures containing only three to five amino acids. A chromatogram with twenty or more amino acids on it would require for the analysis a considerable quantity of enzyme solution and we presume that there would be a tendency for smudging to occur during the reaction on the wet paper which would result in some confusion in distinguishing adjacent amino acids. Auclair & Patton (1950), nevertheless, have used such a method with insect blood. They added the refinement of spraying a duplicate chromatogram with 2-4-dinitrophenylhydrazine to detect the keto acids produced in the reaction.

We have therefore attempted here to simplify the procedure hitherto reported both in the preparation and in the use of the D- and L-amino acid oxidases. We have then applied this method in a preliminary manner to normal human plasma ultrafiltrate, to a hydrolysate of casein, and to seven representative samples of human pathological fluids.

METHODS

Preparation of enzymes

D-Amino acid oxidase was prepared from sheep's kidney cortex by a modification of the procedure of Negelein & Brömel (1939). Our aim was to prepare by as simple a means as possible an active enzyme free, in the amounts to be used, from detectable quantities of amino acids. We therefore omitted stage 2 of Negelein & Brömel's procedure, during which the splitting of the prosthetic group is brought about. In this way we avoided having to prepare the alloxazine-adenine dinucleotide in order to reconstruct the active enzyme. From then on the purification procedure was carried to stage 4, the centrifuged precipitate being dried *in vacuo* over P_2O_5 and then kept in a desiccator over KOH at 4°. When subsequently used, a solution containing 1·5 mg./ml. of enzyme protein (N × 6·25) was made up in 0·067 M sodium pyrophosphate buffer at pH 8·3. The quantity of

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Table 1. Q_{O_2} *of* L-*amino acid oxidase in various snake venoms*

Q_{O_2} = μl. O_2/mg. dry matter/hr.; sodium phosphate buffer (0·067 M, pH 7·2); snake venom, 0·5 mg.; DL-amino acids 2×10^{-5} M; final volume 2 ml. Q_{O_2} was calculated from the O_2 uptake during the first 30 min.

	DL-Leucine		DL-Methionine		DL-Phenylalanine	
	O_2	Air	O_2	Air	O_2	Air
Cobra	131	82	143	112	172	56
Bothrops atrox	—	—	—	—	245	—
Vipera libetina	—	—	—	—	185	—
Russell viper*	← Inactive →					

* From the preparation 'Stypven' of Burroughs, Wellcome and Co.

enzyme solution taken for each test showed no detectable amino acids when analysed by paper chromatography. It was free from catalase and no O_2 uptake could be demonstrated without the addition of substrate. The O_2 uptake/mg. protein/hr. at 37° with 0·05M DL-alanine as substrate varied from 800 to 1300 μl. according to the freshness of the solution, since even at −10° it lost 30–40% of its activity in a week.

Snake venoms were chosen as sources of L-amino acid oxidases (Zeller & Maritz, 1944). Dried venoms from various species were kindly provided by the late Dr C. H. Kellaway and tested for activity. They were stored at room temperature over KOH. The Q_{O_2} varied according to the species and were lower than those reported by Zeller & Maritz. Table 1 summarizes some of our data. It is well known that the range of oxidase activity is wide and unpredictable. On the basis of these results we used *Bothrops atrox* venom in all our later experiments. The venom solutions (2 mg./ml.) kept at 4° or −10° had a remarkable stability and gave reproducible results over a period of weeks. In the amounts used, the solutions contained no detectable concentrations of amino acids. No O_2 uptake was observed without added substrate.

Since no catalase was present in either of the D- or L-amino acid oxidases used here, the O_2 uptake was represented by 1 mole O_2 for 1 mole of substrate.

Substrates

The substrates tested have been casein hydrolysate, plasma ultrafiltrate, pathological samples of cerebrospinal fluid and urine, and made-up mixtures of amino acids.

The casein was hydrolysed with 6N-HCl for 24 hr. at 100°, the hydrolysate evaporated repeatedly to dryness *in vacuo* after additions of water and the residue finally diluted to a concentration corresponding to 3·3 mg. of the original casein/ml.

Both the plasma and cerebrospinal fluid were deproteinized by ultrafiltration through collodion bags (Dent & Schilling, 1949) followed by electrolytic desalting in a micromodification (Dent, 1952) of the apparatus described by Consden, Gordon & Martin (1947). The plasma was finally concentrated to one-fifth of its original volume by evaporation *in vacuo* at 40°. We had confirmed in trial experiments that our L-amino acid oxidase had no action on plasma concentrated without desalting to 0·274 M-NaCl (Zeller, Iselin & Maritz, 1946).

The urine was used as passed.

Incubation

All experiments with pure amino acids and four of the seven with pathological fluids were performed in Warburg flasks in order to follow the O_2 uptake quantitatively and to determine the time at which oxidation was complete.

In each run six flasks (or more if various concentrations of the substrate had to be tested) were set up. In tests with D-amino acid oxidase the main compartment of the flask contained 0·40 ml. of 0·067 M sodium pyrophosphate buffer (pH 8·3) and either the solution to be analysed or a solution of synthetic amino acid as substrate (0·1 ml. of M DL-alanine). In tests with L-amino acid oxidase the main compartment contained 0·25 ml. of 0·067 M sodium phosphate buffer (pH 7·2) and either the solution to be analysed or a solution of L-amino acid as substrate (0·25 or 0·30 ml. of 0·04 M L-tryptophan). The side arm contained 0·5 ml. of the enzyme solution (namely 0·75 mg. of enzyme protein as D-amino acid oxidase or 1 mg. of dry snake venom as L-amino acid oxidase). In control blanks the enzyme was omitted. The final volume of the reaction mixture was made to 2 ml. with water. The centre well contained 0·1 ml. of 20% (w/v) KOH. The gas space contained O_2. The incubation at 37° was extended generally to 3 hr. but in some experiments to 5 or 17 hr.

With the concentration of enzyme used, the theoretical O_2 uptake for the pure amino acids was usually reached in 3 hr. The other substrates usually showed an almost complete stop in O_2 uptake by this time. The contents of each flask were subsequently analysed chromatographically as below.

Chromatographic analysis for amino acids

Two-way paper chromatograms on large squares of filter paper (18 × 22 in.) were always used (Consden, Gordon & Martin, 1944). Phenol was used as first solvent in the presence of NH_3 and HCN vapour and collidine : lutidine in the presence of diethylamine as second solvent (Dent, 1948).

With the casein hydrolysate, a solution containing 510 μg. of original casein was taken for each analysis. With plasma and cerebrospinal fluid the equivalent of 625 μl. of original fluid was taken. The volume of urine taken was that which contained 250 μg. N (Kjeldahl).

An additional one-way chromatogram in *tert.*-amyl alcohol in the presence of diethylamine was also carried out on similar quantities, to distinguish methionine, leucine and isoleucine (Work, 1949).

RESULTS

Casein hydrolysate

The L-amino acid oxidase brought about the complete disappearance from the casein hydrolysate of histidine, leucine, isoleucine, methionine, phenyl-

alanine and tyrosine. In addition, about half of the valine disappeared. These are the amino acids which induce the highest oxygen uptake in experiments with single amino acids (Zeller & Maritz, 1944, 1945). Incubation for 2–3 hr. gave an almost maximal oxidation; very little further oxygen uptake or change in amino acid pattern was induced by continuing to 5 or 17 hr. The observations of Zeller & Maritz (1944) and of Krebs (1935) on the influence of the concentration of the substrate on the enzymic activity of L- and D-amino acid oxidases led us to determine the effect of the L oxidase on amounts of casein hydrolysate containing 815, 1630 and 3260 μg. of original casein. In each case the oxygen uptake was directly proportional to the quantity of substrate added and the final observed pattern of amino acids was the same.

Ultrafiltrate of normal plasma

The L-amino acid oxidase brought about the complete disappearance from the plasma sample of the histidine, isoleucine, leucine, phenylalanine and tyrosine, i.e. all the amino acids detectable in plasma by our methods and known to be oxidized readily by the enzyme. In addition, about half of the valine disappeared. Quantities of plasma assumed to contain 70–140 μg. α-amino N were treated with the enzyme. The oxygen uptake was roughly proportional to the amount of α-amino N. The final pattern of amino acids was the same in each case. There was, however, a slight oxygen uptake with the substrate alone without enzyme. Experiments were also carried out adding L-tryptophan (in amounts containing 75 μg. α-amino N) and L-tyrosine (in amounts containing 25 μg. α-amino N) to the plasma (containing 140 μg. α-amino N). In these cases the enzyme decomposed the added amino acid as well as the above plasma constituents, so there is no evidence that under these conditions appreciable substrate competition for the enzyme was taking place.

In all the above experiments the test simultaneously run with D-amino acid oxidase showed no appreciable oxygen uptake or decomposition of amino acids.

Cerebrospinal fluid

The fluid from two patients with phenylketonuria (phenylpyruvic oligophrenia) has been examined. In the volume (0·5 ml.) taken for chromatographic analysis only phenylalanine and glutamine were detectable as strong spots, the other amino acids being present at much lower concentrations than in plasma. A 2 hr. incubation with L-amino acid oxidase produced in the one case a complete disappearance of the phenylalanine, in the other an almost complete disappearance. From the oxygen uptake the concentrations of amino acid oxidized (calc. as phenylalanine) were 8·1 and 13·3 mg./100 ml., respectively. The D-amino acid oxidase produced no appreciable oxygen uptake and no apparent decomposition of the phenylalanine.

Urine

The urine passed by four patients with Fanconi syndrome was tested. Two of these were adult cases, the other two were children who also showed cystinosis. All the specimens showed gross aminoaciduria. The L-amino acid oxidase treatment resulted in complete disappearance of histidine, isoleucine, leucine, phenylalanine and tyrosine, and also of about half the valine present. These were the only amino acids detectable in the urine that are known to be readily oxidized by the enzyme. The D-amino acid oxidase was applied to one of the adult and to one of the child cases. It had no effect on the amino acids present. Among those easily detectable which, if in the D form, would have been oxidized were (in decreasing order of rate of reaction) proline, isoleucine, phenylalanine, alanine, valine, serine, leucine and tyrosine.

The urine from a case of 'Hart's syndrome' was tested with the L-amino acid oxidase only. This syndrome (E. W. Hart, to be published) has not yet been described clinically, but among its features there is found a gross aminoaciduria of renal origin. The L oxidase was shown to destroy completely the histidine, isoleucine, leucine, phenylalanine, tryptophan and tyrosine, and also over half of the valine present.

DISCUSSION

The method described here for determination of amino acid configuration involves paper-chromatographic analysis of the original mixture before and after allowing it to react with both D- and L-amino acid oxidases. The reactions were mostly carried out in Warburg flasks to check the rate of oxygen uptake and control experiments with the enzymes alone and with added substrate were also run. For routine purposes, however, it is probably unnecessary to do this, since with complicated biological fluids it has been impossible to interpret quantitatively the figures for oxygen uptake. The Warburg technique remains useful only in cases of negative reaction and to detect the end point when reaction is taking place.

As proof of configuration we have found that with isoleucine, leucine, methionine, phenylalanine and tyrosine, all being substances that react with both oxidases, there is obtained a convincing double check, namely that the amino acid is totally destroyed by the one enzyme and quite unaffected by the other. In the case of valine, however, the action with the L oxidase is much slower and tends to be incomplete even after 17 hr. incubation. It

has only been possible to show, therefore, that the destruction of valine in the biological fluids was of the same order as is obtained with the L-valine of a casein hydrolysate and was not effected at all by the D oxidase. We presumed, therefore, that most, if not all, of it was of L configuration. In the case of alanine, histidine, proline and tryptophan, only one or other of the oxidases reacts readily. The evidence of configuration for these substances has to be based only on survival or decomposition by the one enzyme in question. We may further conclude, from the data of Krebs (1935), that a similar single check probably will provide evidence for configuration for further amino acids than the ten mentioned above. These are, in decreasing order of their rates of reaction with the D oxidase: serine, α-aminobutyric acid, cysteine, arginine, aspartic acid and glutamic acid. We have not, however, investigated the reaction under our conditions with the D substrates in question. There is scope for trials of oxidases from other sources, as from *Neurospora crassa* (Horowitz, 1944; Bender & Krebs, 1950), since the amino acid specificity of these is quite different (see also Stumpf & Green, 1944).

The preparation from sheep's kidney cortex of D-amino acid oxidase, sufficiently free of amino acids for our purposes, is now simplified. The L oxidase from snake venom is not, however, likely to be generally available. Unfortunately the commercial Russell viper venom was found to have no L oxidase activity.

When the method was applied to human plasma no evidence for the presence of D-amino acids was obtained. In addition the phenylalanine at elevated concentration (8–13 mg./100 ml. according to the oxygen uptake) in the cerebrospinal fluid in phenylketonuria was found to be all of L configuration. A similar conclusion has recently been reached by Prescott, Borek, Brecher & Waelsch (1949), who used microbiological assays. The urine from four cases of Fanconi syndrome and from one case of 'Hart's syndrome' all showing gross aminoaciduria in the presence of normal plasma levels of α-amino nitrogen likewise appeared to contain only L-amino acids, as far as we could ascertain. This confirms that the aminoaciduria is due to a true lowered renal threshold for the normal blood amino acids (Dent, 1947), and is not due to the presence in the plasma of D-amino acids. These latter are already known to be excreted more easily by the kidney than the L-forms (Silber, Seeler & Howe, 1946; Crampton & Smyth, 1953) and, if present, could have caused an apparent 'renal aminoaciduria' of the type occurring in the Fanconi and other syndromes.

From these preliminary results we therefore conclude that the method is likely to be applicable to most biological fluids. The difficulties we anticipated, namely, inactivation of the enzyme by unknown poisons or by high concentrations of salts, inability of the enzymes to react with minute concentrations of substrate, etc., have not occurred or have been circumvented.

SUMMARY

1. A micro method for the determination of the optical configuration of amino acids in biological fluids is described. In its present form the only common amino acids to which it has been shown to be applicable are alanine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine.

2. The method involves incubation of the fluid with D-amino acid oxidase of sheep's kidney and with L-amino acid oxidase of snake venom. The extent of the reactions is followed by paper-chromatographic analysis.

3. D-amino acid oxidase, free from amino acids and active enough for the present purposes, has been prepared without the need to split off and subsequently to add the coenzyme.

4. The method has been applied to acid hydrolysate of casein, to a human plasma ultrafiltrate, to the cerebrospinal fluid from two cases of phenylketonuria (phenylpyruvic oligophrenia) and to the urine from two cases of Fanconi syndrome (adult type), two cases in children of Fanconi syndrome with cystinosis, and one case of a syndrome involving renal aminoaciduria, not yet fully described clinically. No evidence was obtained of the presence in these fluids of D forms of the above ten amino acids.

We should like to record our grateful thanks to the late Dr C. H. Kellaway, F.R.S., for gifts of snake venoms, and to Miss D. I. Fowler for some technical assistance. The work was carried out during the tenure by one of us (E. B.) of a Fellowship from the British Council and by the other (C. E. D.) of a Foulerton Fellowship from the Royal Society.

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Respiration Rate of Bacteria as a Function of Oxygen Concentration

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The respiration rate of an organism is measured by determining the rate at which it absorbs oxygen. It is usual to relate this value to some measurement of the size of the organism such as weight or surface area. Variations in the rate of respiration have been determined in this work, so absolute measurements will not be mentioned. In the absence of oxygen, respiration cannot take place and it is known that when the concentration of oxygen surrounding the organism is progressively reduced, the respiration rate falls as the concentration becomes sufficiently low. In this paper is described a study of the respiration rate of some bacteria as a function of oxygen concentration.

When bacteria are suspended in a medium containing a suitable supply of nutrient they use up the oxygen in solution. If the solution is exposed to the air, oxygen will go into solution, tending to bring the system back to equilibrium. After a time a steady state is set up when the rate of solution of oxygen equals the rate at which the organisms are respiring. By using a manometric device the rate of solution can be measured and thus the respiration rate. It is possible to determine the concentration of oxygen in solution by techniques of this sort. One method is to employ very small numbers of bacteria so that the rate of uptake of oxygen is so slow that in the steady state the oxygen in solution is very nearly in equilibrium with that in the gas phase.

In papers published some years ago (Warburg & Kubowitz, 1929; Meyerhof & Schulz, 1932; Shoup, 1929; Cook, 1930) manometric methods of determining the oxygen concentration at which yeast and various bacteria respire at half their maximum rates were described. The figures so obtained were for a few organisms only and there appeared to be some disagreement, even allowing for the large temperature coefficients. It seemed advisable to redetermine this oxygen concentration for a number of organisms in the hope of finding some fundamental relationship so that the measurements could be predicted for any organism at any temperature and pH.

An attempt was made to repeat the previous manometric techniques and an improved method suggested by Dr D. Herbert was also tried. It was soon apparent that while these methods might yield results in the hands of highly skilled workers, in fact they involved working at the limits of usefulness of manometric techniques where diffusion from the gas to the liquid phase becomes limiting (Dixon, 1943). It was therefore decided that direct measurement of the oxygen concentration in solution by a polarographic method should be tried.

The use of the conventional dropping-mercury electrode was first investigated. It was found that a slowing of the respiration rates of bacterial suspensions at low concentrations of oxygen could be shown, but that this occurred at the limits of sensitivity of the instrument.

Harris & Lindsey (1948) described a vibrating platinum electrode which gave very much higher diffusion currents for the same concentration of oxygen when compared with the dropping-mercury electrode. This was tried, but for mechanical reasons it could not be used conveniently in a cell free from a gas phase.

During a visit here, Prof. Chain suggested a very ingenious rotating platinum electrode, which easily overcame this difficulty. Several experiments showed that measurements of the concentration of oxygen at which bacteria respire at half their maximum rates could be obtained with this apparatus. Duplicate determinations, however,

varied rather badly and investigation showed that this was due to overlapping of the oxygen and hydrogen waves. Kolthoff & Jordan (1952) demonstrated that there was a wider separation of two waves using gold instead of platinum. A gold electrode was employed and gave results agreeing well with one another, and this type of electrode was used for all the determinations reported here.

MATERIALS AND METHODS

Solutions. Potassium chloride (0·1 M) was prepared from Polaritan Reagent (Hopkin and Williams Ltd.) using distilled water that had been further purified by passage through a column of Amberlite. MB—1 (British Drug Houses Ltd.).

Phosphate buffer 0·1 M pH 7·3 was prepared from AnalaR phosphoric acid, Polaritan KOH (Potassium Hydroxide Pellets Polaritan) and pure water by the method described by Slater (1952) so as to be free of pyrophosphate.

A 1 % solution of glucose in a mixture of equal volumes of the above solutions was used as the suspending medium in all the experiments. The pH was 7·3 except when otherwise stated, when it was altered by adding Polaritan KOH till the required pH was obtained.

Organisms. In general the organisms were grown on tryptic meat-digest agar at 37°. The overnight growth was usually sufficient but some required 36 hr. *Micrococcus candicans*, *Acetobacter suboxydans* and *Azotobacter indicum* grew best at 25°. Some of the cultures of *Bacillus megatherium* were grown on this medium to which 1·5 % glycine or 1 % LiCl had been added in order to produce cells larger than normal.

Acetobacter suboxydans was grown on malt wart liquor with added chalk.

Azotobacter indicum was grown on a medium containing KH_2PO_4 (0·1 %), $MgSO_4$ (0·02 %), $FeSO_4$ (0·02 %), Na_2MoO_4 (0·0002 %), glucose (0·5 %), sucrose (1·0 %), agar (2 %) and water to 100 %. The organisms were washed off the agar with the suspending medium. The pig- and ox-heart preparations were made by the method of Slater (1949). The baker's yeast was purchased locally.

Method. The polarographic method used continuously records the concentration of O_2 in solution. The gold electrode (Fig. 1) consists of a small sphere on the end of a wire sealed into a piece of soda-glass tubing, and electrical connexion between this and the wire to the polarograph is made with Wood's metal. The electrode is inserted into the cell through a hole in a rubber disk. The fit of the electrode into the disk, and of the disk into the cell, is so tight that leakage of air cannot occur even when the pressure difference between the cell and the atmosphere is 760 mm. of mercury. The other end of the electrode is connected by a piece of rubber tubing to an iron rod which engages through a journal bearing concentrically situated in a brass disk rotated at 1300 rev./min. by a constant-speed motor. The lower end of the cell is closed by a sintered glass disk, on top of which is a layer of 4 % agar in saturated KCl. This end is immersed in a solution of saturated KCl which is connected with a saturated calomel electrode by means of an agar bridge. This electrode and the bridge must be large enough not to polarize with the passage of 150 μA. In practice a 3 lb. bottle of 10 cm. diameter and tubing of 1 cm. bore satisfied this requirement. The cell is filled with the suspending medium, all air bubbles being removed. The volume of liquid in the cell is about 20 ml.

The gold electrode is made the cathode, and the calomel cell the anode in their connexions with a Cambridge pen-recording polarograph. The model used is type no. 91331 and it has a sensitivity range of 400 to 0·4 mm./μA.

Theoretical basis of method. When a constantly increasing potential is applied across the electrodes, O_2 is reduced at the cathode, and a current flows which is proportional to the rate of reduction. It is postulated that rapid rotation of the electrode results in the retention of a very thin layer of stationary liquid around the moving part, while the bulk of the liquid is fairly uniformly mixed. If now a sufficiently great potential difference is applied to reduce all the O_2 at the gold surface, a diffusion gradient will be set up across the stationary layer. The rate at which O_2 diffuses through this layer will be proportional to its concentration in the bulk of the solution, and thus the current flowing through the cell will be proportional to the concentration of oxygen in solution.

The current flowing will be proportional to the area of the electrode and inversely proportional to the thickness of the stationary layer. These two variables were investigated in order to find the optimum conditions. Increasing the rate of rotation increased the diffusion current, but above 1000 rev./min. this increase tends to a plateau, and in practice a speed of 1300 rev./min. was found convenient, as changes in speed produce relatively small changes in current at this point. Theoretically, increasing the size of the gold sphere should increase the diffusion current indefinitely. In practice the internal resistance of the polarograph becomes the limiting factor, and it was found convenient to use an

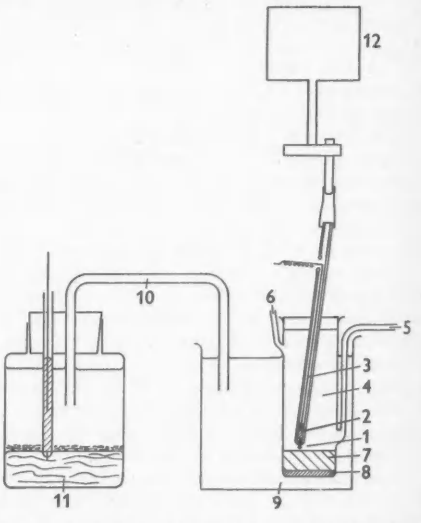

Fig. 1. Diagram of the apparatus. 1, gold electrode; 2, Wood's metal; 3, wire to polarograph; 4, cell containing suspending medium; 5, inlet; 6, outlet; 7, 4 % agar in sat. KCl; 8, sintered glass; 9, sat. KCl; 10, agar bridge; 11, saturated calomel half-cell of 10 cm. diam.; 12, constant-speed motor.

electrode of 1·5 mm. diameter, which gives a current of 150 μA when the O_2 concentration is that of an air-saturated solution. This current gives full-scale deflexion on the recorder of the instrument at minimum sensitivity.

The selection of the potential at which all the O_2 at the gold surface is reduced gave some difficulty. When a constantly increasing potential is applied across the electrodes, the current flowing increases in such a manner as to produce two steps or waves (Fig. 2).

Electrode reactions. The reactions giving rise to these waves differ from those which produce the two waves when O_2 is reduced at the dropping-mercury electrode. In the latter case the first wave is due to the reduction of O_2 to H_2O_2 and the second to the reduction of H_2O_2 to OH^- (Kolthoff & Miller, 1941). Laitinen & Kolthoff (1941) studied the two waves formed when O_2 is reduced at the platinum electrode. They found that the first wave is due to the reduction of O_2 to H_2O_2 and the second to the reduction of H^+ to H_2. The large hydrogen overvoltage found with mercury does not occur with platinum and so the hydrogen wave occurs at a very much more negative potential and presumably masks the peroxide wave. In their discussion on the reactions occurring at the gold electrode Kolthoff & Jordan (1952) stated that they occur as follows:

$$O_2 + e \rightarrow O_2^-, \quad (1)$$

$$O_2^- + e \rightarrow O_2^{2-}, \quad (2)$$

$$O_2^{2-} + 2H^+ \rightarrow H_2O_2. \quad (3)$$

They then state that if H_2O_2 is added to the solution the following reactions then take place:

$$O_2^- + H_2O_2 \rightarrow OH^- + O_2 + OH, \quad (4)$$

$$OH + e \rightarrow OH^-. \quad (5)$$

These authors utilized these second reactions to devise a method of exalting the diffusion current of oxygen so that very small quantities of O_2 could be determined. However, it seemed that even if no H_2O_2 was added, that which is formed in reaction (3) might react in the same way. If all the H_2O_2 reacts in this way then the overall reaction will be

$$O_2 + 4e + 2H^+ \rightarrow 2OH^-. \quad (6)$$

If, on the other hand, none of the H_2O_2 undergoes this further reaction, the overall reaction will be

$$O_2 + 2e + 2H^+ \rightarrow H_2O_2. \quad (7)$$

It seemed to be desirable to determine the overall reaction occurring at the surface of the electrode described here. If it is reaction (7), then the H_2O_2 formed might interfere with bacterial metabolism, or if the organism produced catalase this might result in an increase of the diffusion current for the same concentration of O_2.

In order to test whether or not the H_2O_2 formed at the electrode undergoes further reduction, the coulombs passing for each g.mole of oxygen disappearing were determined. In the first experiment the apparatus was set up as shown in Fig. 1. Some difficulty arose over the loss of O_2 by diffusion into the agar layer. Normally this loss is insignificant, but it is comparable in amount to that being reduced at the electrode. When a correction had been made for this loss it was found that 3·8 F (coulombs) had passed for each g.mole of oxygen reduced. As the correction for the loss of oxygen by diffusion was rather large, it was considered desirable to devise an experimental arrangement in which this loss could not occur. This was done by replacing the agar by a pool of mercury which then acted as the anode. Electrical contact with this pool was made by immersing the cell in a second pool of mercury; the pores of the sintered glass disk being filled with mercury. Connexion with the polarograph was made by placing a copper wire in this second pool. It was found in this experiment that 3·69 F (coulombs) passed for each g.mole of oxygen reduced.

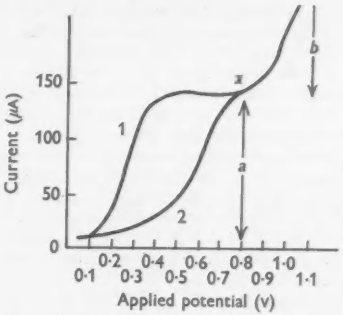

Fig. 2. Polarograms obtained by applying a progressively increasing potential between the gold cathode and the anode. As the potential increases the current increases in the manner shown in these curves. The first wave, (*a*), is due to the reduction of oxygen and the second, (*b*), to the reduction of hydrogen ions. Curve 1 is obtained after the initial preparation (Kolthoff & Jordan, 1952) and curve 2 after the application of about 0·8 v for 30 min. The potential at *x* where the two curves begin to overlap is the one chosen for current measurements. It will be seen that if a point on the first curve to the left of *x* were chosen, the diffusion current would appear to diminish with time.

These experiments show that most if not all the H_2O_2 is reduced at the electrode. Chemical determination of H_2O_2 free in the solution in the cell after experiments of this kind showed that less than one-tenth of the amount formed by reaction (3) remained. This quantity is much too small to interfere with bacterial metabolism. The addition of excess of catalase did not affect the diffusion current.

From these results it appears that the overall reaction is not stoicheiometric and the possibility that the number of faradays passing for each g.mole of oxygen reduced might vary with different concentrations of oxygen must be considered. If this occurs the diffusion current may not vary linearly with the O_2 concentration. In calibration experiments it has been found that by plotting the diffusion current against O_2 concentration determined by Van Slyke's (Peters & Van Slyke, 1948) and Winkler's (Snell & Snell, 1949) methods a straight line is obtained running through the origin. The range of concentrations used was 10^{-3}–10^{-7} M.

If the electrode is allowed to rotate for 30 min. at an applied potential of just less than 1 v and the potential is then varied between 0 and 1 v, the oxygen wave will be found to have been shifted towards the hydrogen wave. The two waves are still distinct enough to enable a potential to be selected at which all the O_2 but none of the hydrogen ions were reduced, and thus the diffusion current is unaltered It is important to 'age' the electrode in this way before beginning any determinations. The ageing process is associated with a change in the surface appearance of the electrode, which loses its brightness and becomes matte. Usually the potential selected was 0·7 v.

The current flowing at 0·7 v is not entirely due to the reduction of oxygen; the diffusion current of the reduction of some metal ions, the condenser current and the residual current all contribute. If the oxygen is all removed by a reducing agent such as Na_2SO_3 or a respiring biological system these other currents can be measured. In practice, using Polaritan reagents, they are very small.

It appeared probable that some experiments might take a long time to perform, and so it was necessary to determine the stability of the diffusion current. The apparatus was run for several periods of 3 days and the greatest change in one period was a fall of 5%, but in the others the change was less than 2%. The changes were linear with time so corrections can be made.

Errors can arise from the loss of O_2 by reduction at the cathode and by diffusion into the agar and the rubber disk, but in the time during which individual determinations are made these errors are negligible.

The diffusion current has a temperature coefficient of about 2%/1°, approximately the same as that of the diffusion coefficient of O_2 in water. Thermostatic temperature control is therefore essential. The temperature of the room used in this department is kept so constant that temperature variations in the apparatus, without the use of special precautions, were less than 0·1° during the course of any one experiment. The experiments at higher temperatures were conducted in a hot room.

Experimental protocol. When a suspension of organisms is introduced into the cell the O_2 is all used up and the diffusion current falls to zero. In this way an initial calibration point is obtained. The next stage is to introduce some more O_2 in solution and record its disappearance at a higher instrumental sensitivity. It is found that the O_2 is used up at a constant rate at first and later more slowly, the curve finally becoming horizontal when no O_2 remains (Fig. 3). The paper of the recorder is carried on a curved carriage with the pen arm pivoted at the centre of curvature. Thus a constant rate of uptake produces a straight line. The maximum slope is now measured and a line of half this slope is drawn so as to touch the curve. The distance between the point of contact and the bottom of the curve (zero O_2) is a measure of the O_2 concentration at which the bacteria respire at half their maximum rate.

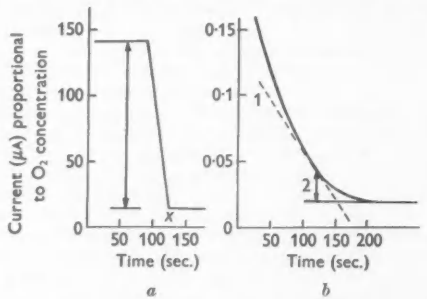

Fig. 3. The effect on the diffusion current when a bacterial suspension is added to the cell. In (*a*) the solution is initially saturated with O_2 at atmospheric pressure and finally it is O_2 free. In this way the current flowing at a known concentration of oxygen is determined and the apparatus calibrated. Fig. 3 (*b*) is similar to (*a*), except that it is obtained at a higher instrumental sensitivity and represents a 1000-fold magnification of region *x* of curve (*a*). 1, line drawn to touch curve, slope of line half maximum slope of experimental curve; 2, Km, the concentration of oxygen at which the bacteria respire at half their maximum rate.

In practice it was found convenient to adjust the amount of bacteria present so that the initial slope of the curve was about 2, and the sensitivity selected was such as to give a measurement of the half rate less than 10% of full-scale deflexion.

In previous work the object has been to determine the O_2 concentration at which bacteria respire at half their maximum rate. The method described above produced a continuous recording of the O_2 consumption of a bacterial suspension at continually varying O_2 concentration, so it seemed that more information might be obtained by inspection of the whole curve rather than consideration of one point only. The results actually obtained from the recorder of the polarograph consist of a plot of O_2 concentration against time, and the shape of these curves appeared to be similar to substrate concentration × time curves for enzymes acting on their substrates. If this is so the experimental relationship between O_2 concentration and time ought to fit the Michaelis-Menten relationship. This is normally stated in the form $-\frac{dS}{dt}=\frac{k'S}{Km+S}$, but for convenience in the consideration of the relationship of concentration and time it may be integrated, when it yields

$$\frac{S_0-S}{t}=k'+\frac{Km}{t}\times\ln\frac{S}{S_0},$$

where S_0 = the initial oxygen concentration;

S = the oxygen concentration at time t;

k' = the maximum respiration rate;

Km = the Michaelis constant (i.e. the substrate concentration giving $-ds/dt=\frac{1}{2}k'$).

When $\frac{S_0-S}{t}$ is plotted against $\frac{1}{t}\times\ln\frac{S}{S_0}$ an approximately straight line of slope Km is obtained. Values of Km so obtained agree well with the concentrations of oxygen at which the bacteria respire at half their maximum rate as read from the experimentally derived curves.

Alternatively, by determining values of t from this form of the integrated equation

$$t=\frac{S_0-S}{k'}+\frac{Km}{k'}\times\ln\frac{S_0}{S}$$

at various values of S read off 8-fold enlargements of the experimental curves obtained from any of the organisms, Km and k' being similarly derived, and then plotting them against the appropriate values of S, curves are obtained which fit the experimental

ones (Fig. 4). The fit is relatively poor at the bottom of the curves, the experimental curve lagging behind the theoretical curve by 0·3 mm. at the worst point. This could be due to friction between the pen and the paper.

Thus it is not unreasonable to conceive of the bacterium standing in the same relationship to O_2 as an enzyme does to its substrate, and therefore the concentration of oxygen at which it respires at half its maximum rate may be designated the *Km*.

Since the Michaelis constant is theoretically an inverse measure of the affinity of an enzyme for its substrate, by this analogy the concentration of oxygen at which bacteria respire at half their maximum rate is an inverse measure of their affinity for oxygen.

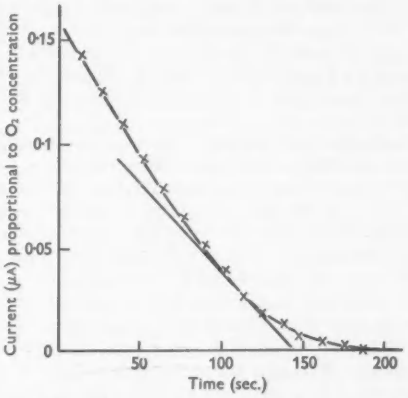

Fig. 4. The continuous curve is obtained experimentally. The straight line touching it has half the maximum slope of the curve. The crosses are points calculated from the equation $t = \frac{S_0 - S}{k'} + \frac{Km}{k'} \times \ln \frac{S_0}{S}$.

RESULTS

In Table 1 are a number of results for various organisms. Measurements on yeast and pig- and ox-heart preparations have been included for comparison. The heart preparations were oxidizing succinate and not glucose as was the case with the bacteria.

It will be noted that there are fairly wide variations, the larger organisms having a larger *Km*. At this stage in the experimental work it was realized that size and *Km* were related. It looks as though the relationship is of the form $Km = Ad^x$, where A is the proportionality factor, d is the cell diameter and x is a constant which is a function of cell shape. This theory was tested by plotting log *Km* against log d. The value of d was obtained from standard textbooks of bacteriology, e.g. Bergey (1948), Topley & Wilson (1946) except in the case of the *B. megatherium* cultures in different media where the values of d were estimated by comparison with one another. More precise measurements would have been desirable, but the resultant plot was so nearly a straight line of slope 2·6032 and intercept of −7·0460 that it was felt there was something worth looking into. The fiduciary limits of the log *Km* calculated from the cell diameter are ±0·2605, which are sufficiently narrow to permit calculations of *Km* from cell size accurately enough for the practical consideration of growing organisms.

It is obvious that a variation of *Km* which is a function of size is not likely to be due to a variation in the affinity of the respiratory enzymes for oxygen. An explanation based on diffusion seems more probable. Attempts were therefore made to measure the *Km* of cell-free preparations containing the respiratory system in contact with the bulk of the suspending fluid. In the intact cell these two are presumably separated by a layer of cell substance across which there is a diffusion gradient.

Table 1. *The* Km *values of various organisms showing the effect of cell size*

The *Km*'s of baker's yeast and heart-muscle preparations are shown for comparison. The bacteria and yeast were suspended in a solution of 1% glucose in a mixture of equal parts of 0·1 M-KCl and 0·1 M phosphate buffer of pH 7·3. For the heart-muscle preparations the glucose was omitted and the solution instead made 0·004 M with respect to succinate.

Organism	Diameter (μ)	Temperature (°)	*Km* (molar O_2) ± coefficient of variation
Aerobacter aerogenes	0·6	19·0	$3{\cdot}10 \times 10^{-8} \pm 9{\cdot}0\%$
Micrococcus candicans	0·5	20·2	$1{\cdot}10 \times 10^{-8} \pm 9{\cdot}1\%$
Bacillus megatherium	2·0	19·2	$5{\cdot}97 \times 10^{-7} \pm 3{\cdot}3\%$
B. megatherium (glycine)	4·0	20·6	$3{\cdot}12 \times 10^{-6} \pm 7{\cdot}0\%$
B. megatherium (LiCl)	2·4	20·0	$7{\cdot}07 \times 10^{-7} \pm 1{\cdot}8\%$
Azotobacter indicum	1·6	19·6	$3{\cdot}00 \times 10^{-7} \pm 7{\cdot}3\%$
Acetobacter suboxydans	2·7	19·2	$1{\cdot}57 \times 10^{-6} \pm 4{\cdot}5\%$
Serratia marcescens	0·7	18·8	$3{\cdot}60 \times 10^{-8} \pm 5{\cdot}0\%$
Escherichia coli	0·6	19·2	$2{\cdot}22 \times 10^{-8} \pm 3{\cdot}2\%$
Baker's yeast	—	19·0	$6{\cdot}45 \times 10^{-7} \pm 3{\cdot}3\%$
Pig-heart preparation	—	17·3	$1{\cdot}86 \times 10^{-8} \pm 6{\cdot}4\%$
Ox-heart preparation	—	19·8	$2{\cdot}39 \times 10^{-8} \pm 5{\cdot}4\%$

Table 2. *Comparison of the* Km *values of intact cells and cell-free preparations made from them*

Both the cells and the cell-free preparations were suspended in 1% glucose in a mixture of equal parts of 0·1 M-KCl and 0·1 M phosphate buffer pH 7·3.

Organisms	Temp. (°)	*Km* (molar O_2) Intact cells	*Km* (molar O_2) Cell-free preparation
Aerobacter aerogenes	18·0	$4{\cdot}01 \times 10^{-8}$	$3{\cdot}83 \times 10^{-8}$
Bacillus megatherium	18·8	$7{\cdot}13 \times 10^{-7}$	$2{\cdot}87 \times 10^{-8}$
B. megatherium (glycine)	18·2	$1{\cdot}34 \times 10^{-6}$	$3{\cdot}68 \times 10^{-8}$

Table 3. *Effect of temperature on the* Km *values of some bacteria and a heart muscle preparation*

The bacteria were suspended in 1% glucose in a mixture of equal parts of 0·1 M-KCl and 0·1 M phosphate buffer pH 7·3. For the heart-muscle preparation the glucose was omitted and the solution instead made 0·004 M with respect to succinate.

Organism	Temp. (°)	*Km* (molar O_2)
Aerobacter aerogenes	19·0	$3{\cdot}10 \times 10^{-8}$
	35·0	$3{\cdot}72 \times 10^{-7}$
Bacillus megatherium	21·2	$9{\cdot}02 \times 10^{-7}$
	32·2	$2{\cdot}77 \times 10^{-6}$
B. megatherium (glycine)	20·2	$1{\cdot}21 \times 10^{-6}$
	34·0	$3{\cdot}57 \times 10^{-6}$
Ox-heart preparation	19·8	$2{\cdot}39 \times 10^{-8}$
	33·2	$4{\cdot}10 \times 10^{-8}$

Using the method of Tissières (1952) to make the cell-free preparations, experiments were conducted on organisms at both ends of the size scale. It will be seen from the results given in Table 2 that the *Km*'s of the cell-free preparations are approximately the same for the organisms investigated and that they are not very much less than the *Km* of the smallest. This indicates that the *Km* of the small organisms is not appreciably affected by diffusion whereas in the larger ones it is a most important factor. This would be expected to produce a marked affect on the temperature coefficient of *Km*. Determinations of the *Km* of *Aero. aerogenes* at a higher temperature shows there is a 12-fold increase with a temperature rise of 16°. If the temperature coefficients of the *Km*'s of the respiratory systems of *Aero. aerogenes* and *B. megatherium* are the same and the temperature coefficient of the diffusion constant of oxygen through bacterial substance is taken as 2% per degree it is possible to calculate that a rise in temperature of the order of 15° would produce a rise in the *Km* of intact *B. megatherium* of 3·6-fold. Experimentally this rise was found to be 3-fold (Table 3).

The effect of pH change on *Km* was determined in the case of *Aero. aerogenes*. Determinations at pH's lower than 7 are impracticable for polarographic reasons, but determinations at pH's 8 and 9 showed a small but not significant rise with increasing pH. Presumably the pH of the contents of the cell is not much affected by a change in the pH of the suspending medium.

DISCUSSION

Despite the wide variations in the *Km*'s of different organisms and in their temperature coefficients, all the oxygen concentration/time curves fit the theoretical ones. In the larger organisms there is, however, a concentration gradient between the bulk of the liquid and the location of the respiratory system.

It is tempting to visualize the oxygen concentration in the bulk of the liquid falling in parallel with that in the interior of the cell. To demonstrate this it would be necessary to show that the oxygen concentration both outside and inside reach the appropriate *Km* values at the same time. This has not yet proved possible for technical reasons.

If some information can be obtained about the distribution of the respiratory system within the cell it should be possible to calculate the diffusion coefficient of oxygen through cell substance by the methods of Nicholson & Roughton (1951). The fact that *Km*'s of both intact *Aero. aerogenes* and the cell-free preparation from it are so nearly the same suggest that there is a very small diffusion gradient between the bulk of the suspending fluid and the respiratory system, whereas in the case of *B. megatherium* this gradient is much larger. This suggests there is a fairly uniform distribution of the system in *Aero. aerogenes* while in *B. megatherium* the difference in *Km*'s could be due to a localization of the system in the centre of the cell.

Investigations into the nature and distribution of the respiratory system in bacteria are being conducted along the lines suggested above.

It will be seen that the *Km*'s of cell-free preparations and mammalian mitochondria are substantially the same although there are differences in structure and in the cytochrome systems. There is however a big difference in the temperature coefficients (Table 3).

In Table 1, yeast, the largest organism, has not got the highest *Km*. This means it does not fit the equation $Km = Ad^x$ and thus the expression is not generally valid.

SUMMARY

1. A polarographic method of measuring and continuously recording the concentration of oxygen in solution down to the order of 10^{-9}M has been described.

2. This method has been used to determine the respiration rate of bacteria as a function of oxygen concentration.

3. The results so obtained have shown that the respiration rate of bacteria is related to the oxygen concentration by the Michaelis-Menten equation, and the characteristic *Km*'s of a number of organisms have been determined.

4. The effect of temperature and pH changes on the *Km* have been investigated.

5. Reasons for the differences in the *Km*'s of different organisms have been discussed and an empirical equation relating *Km* to the size of the organism has been established.

I wish to thank Dr D. Herbert, at whose suggestion this work was undertaken, for advice and criticism. I am indebted to Prof. Chain for very kindly describing to me the type of rotating electrode devised by Dr G. Gualandi. I am very grateful to Dr D. W. Henderson for his enthusiastic support and encouragement, to Mr L. J. Bardwell and Miss B. J. Clarke for technical assistance and to Mr S. Peto for assistance with the statistics. Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this communication.

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The Role of Magnesium in the Hydrolysis of Sodium Pyrophosphate by Inorganic Pyrophosphatase

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Inorganic pyrophosphatase has been found to be widely distributed in living organisms. It has been demonstrated in various organs and in tissue fluids (Kay, 1928; Lohmann, 1933; Roche, 1931; Minniti, 1939; Nagana & Menon, 1948; Gordon, 1950; Bloch-Frankenthal & Back, 1951). Bauer (1937), who showed it to be present in yeast, obtained it free from phosphomonoesterase. More successful attempts at its purification from yeast were made by Bailey & Webb (1944) and by Heppel & Hilmoe (1951). Kunitz (1952) obtained it from the same source in crystalline form.

Pyrophosphate, the specific substrate of this enzyme, was found in baker's yeast (Lindahl & Lindberg, 1946), insect tissue (Heller, Karpiak & Zurikowa, 1950), and in moulds (Mann, 1944). Kornberg (1950) reported recently on the production of pyrophosphate in the reversible synthesis of diphosphopyridine nucleotide from nicotinamide mononucleotide and adenosine triphosphate (ATP) in the presence of hog-liver or beer-yeast preparations. He also proposed a mechanism for pyrophosphate accumulation in tissues and fungi. The enzymic splitting of pyrophosphate from ATP has also been demonstrated (Hitchings & Fuller, 1939; Zeller, 1950; Heppel & Hilmoe, 1953).

Pyrophosphatases, so far described, are dependent upon the presence of magnesium for their ability to catalyse the hydrolysis of inorganic pyrophosphate according to the reaction:

$$Na_4P_2O_7 + H_2O \rightarrow 2Na_2HPO_4,$$

or

$$HP_2O_7^{3-} + H_2O \rightarrow 2HPO_4^{2-} + H^+$$

(Bailey & Webb, 1944).

In the absence of this activator no orthophosphate is formed. Lohmann (1933) found that the rate of hydrolysis of sodium pyrophosphate by muscle or liver extract in the presence of magnesium increases with the molar ratio of magnesium chloride to sodium pyrophosphate until an optimum is reached when this ratio is about 2 (see also Gilmour & Calaby, 1953). Bailey & Webb (1944) arrived at the ratio 5 with their purified preparation at pH 7·2. According to Bauer (1937) the optimum ratio of magnesium to pyrophosphate depends both on pH and the concentration of sodium pyrophosphate in the digestion mixture, the ratio increasing considerably with the decrease in pH and also with the decrease in pyrophosphate concentration. Kunitz (1952) reports that the ratios of magnesium concentration to those of pyrophosphate where full activation was reached with crystalline pyrophosphatase were approximately 1 at pH 7·2 and over 100 at pH 5·3.

The high concentrations of magnesium required for optimum activation could hardly be explained in the light of accepted concepts, and some further investigation into the matter seemed desirable. The present paper, which forms part of a study on erythrocyte pyrophosphatase, reports experiments with a purified preparation from rat erythrocytes.

METHODS

Glass-distilled water was used in all experiments and in the preparation of the enzyme.

Preparation of pyrophosphatase eluate. The rats were anaesthetized with ether and bled. The blood collected from the abdominal artery and vein was defibrinated by stirring with a glass rod, and centrifuged. The erythocytes were washed twice with saline and haemolysed by adding water up to twice the volume of the original defibrinated blood. The haemolysate thus obtained was kept at 0° overnight and the precipitate centrifuged off. The supernatant was returned to the refrigerator and used as required within 10 days of its date of preparation.

For the preparation of the eluate 1 ml. of haemolysate was diluted with 2·5 ml. of water and shaken with 1·5 ml. calcium phosphate gel (prepared according to Kunitz, 1952) for 5 min. After centrifuging, the precipitate was washed with water and incubated for 30 min. with 2 ml. of an acidified 0·5% sodium pyrophosphate solution (50 mg. $Na_4P_2O_7$, $10H_2O$ + 8 ml. H_2O + 2 ml. 0·1 N-HCl). The precipitate was centrifuged off and the supernatant was filtered and dialysed in cellophan tubing (Visking) against glass-distilled water, for 20 hr. at least, in the refrigerator. The eluate of erythrocyte pyrophosphatase thus obtained requires the presence of magnesium for catalysing the hydrolysis of pyrophosphate into two molecules of orthophosphate. It forms a clear and absolutely colourless solution almost as rich in activity as the original haemolysate of the same dilution. It is inactive towards glycerophosphate and thiamine pyrophosphate. The properties of erythrocyte pyrophosphatase in the form of the eluate, which will be described in detail elsewhere, conform in general with those of yeast pyrophosphatase. The eluate retains its activity for several days when kept in the refrigerator under a thin layer of toluene. It was used for the experiments on the day of its preparation or on the day after.

Buffer. Veronal/acetate (Michaelis, 1931) was used to buffer the digestion mixtures throughout these experiments. The ingredients of this buffer slightly inhibit the erythrocyte pyrophosphatase according to Nagana (1950), but since their concentrations remain constant throughout the whole pH range of the buffer, it was thought to be preferable to veronal buffer. In this latter buffer, veronal concentration varies with the pH, and its use might lead to false conclusions by attributing results caused by variations in inhibitor concentration to variations in pH and vice versa.

Substrate. Only freshly prepared solutions of sodium pyrophosphate ($Na_4P_2O_7$, $10H_2O$) C.P. (Baker's analysed) were used in all the experiments.

Magnesium chloride. A 10% stock solution of $MgCl_2$, $6H_2O$ (C.P.) was prepared and its concentration determined volumetrically according to Handy (Scott, 1939). Dilutions were prepared as required from this stock solution.

Pyrophosphatase activity measurement. Standard digestion mixture for a total volume of 10 ml.: 6 ml. veronal/acetate buffer; 0·6 ml. 1% $Na_4P_2O_7$, $10H_2O$ ($2{\cdot}24 \times 10^{-2}$ M); 1 ml. approx. 1% $MgCl_2$, $6H_2O$ ($5{\cdot}13 \times 10^{-2}$ M); 0·5 ml. pyrophosphatase eluate (2·5 times diluted immediately before use). The mixture was incubated at 37°.

Estimations. The pH of digestion mixtures was determined in every case. All pH determinations were carried out with the glass electrode, using the Beckman pH Meter (model G).

Phosphate concentration was determined by the method of Fiske & Subbarow (1925) as modified by Lohmann & Jendrassik (1926). The digestion mixtures were treated with an excess of trichloroacetic acid solution (5 vol. of a 7%, w/v, solution), filtered if necessary, and samples taken for the phosphate determination. Colorimetric determinations were carried out in a Klett-Summerson Photoelectric Colorimeter, using number 66 red filter spectral range 640–700 mμ.

RESULTS

Magnesium chloride is involved in the enzymic hydrolysis of sodium pyrophosphate and is a precipitating agent for that salt. Precipitation begins when the molar ratio of $MgCl_2$ to $Na_4P_2O_7$ attains the value 1. At ratios lower than 1 a soluble complex salt is obtained containing the bivalent metal in the anion (Rosenheim & Triantaphyllides, 1915; Chebuliez & Bretagna, 1949). The composition of the complex anion in solution corresponds to the formula $(MgP_2O_7)^{2-}$ (Chebuliez & Bretagna, 1949; Rogers & Reynolds, 1949), but its salt has not been isolated. This complex anion has a limited stability even in solution, and some time after being formed (hours or minutes, depending upon concentration) at ordinary temperature—sooner at higher temperatures and immediately on boiling—it decomposes and precipitation occurs, the precipitate remaining insoluble even after cooling. The composition of the precipitate seems to vary with the precipitation conditions (Bassett, Bedwell & Hutchinson, 1936).

Table 1. *The effect of* $MgCl_2$ *concentration on the stability of its solution in* $1{\cdot}345 \times 10^{-3}$ M-$Na_4P_2O_7$ *at* 37°

$MgCl_2$ concentration (10^{-3} M)	Ratio: $Mg^{2+}/P_2O_7^{4-}$	State of solutions after incubation (hr.)				
		0·5	1	1·5	2·5	4·5
0·26–2·05	0·19–1·53	Clear	Clear	Clear	Clear	Clear
2·57	1·91	Clear	Clear	Clear	Clear	Precipitate
3·08	2·29	Clear	Clear	Clear	Clear	Precipitate
4·10	3·05	Clear	Clear	Turbid	Precipitate	Precipitate
5·13	3·81	Clear	Clear	Turbid	Precipitate	Precipitate
7·18	5·34	Turbid	Precipitate	Precipitate	Precipitate	Precipitate
10·3	7·6	Precipitate	Precipitate	Precipitate	Precipitate	Precipitate

Preliminary experiments to determine the optimum concentrations of $MgCl_2$ and $Na_4P_2O_7$ in the digestion mixtures were performed. As a result of these experiments it was found that on adding $MgCl_2$ to a $1{\cdot}345 \times 10^{-3}$ M solution of $Na_4P_2O_7$, a ratio of $MgCl_2$ to $Na_4P_2O_7$ of approximately 5 could be attained without any precipitation occurring for hours. On incubating the mixtures at 37°, turbidity began to appear in the solution with nearly the highest $MgCl_2$ content ($5{\cdot}13 \times 10^{-3}$ M) only after an hour (Table 1). Another effect of this complex formation between $MgCl_2$ and $Na_4P_2O_7$ is the resulting decrease in the pH of the mixture. This phenomenon has also been observed in the case of phosphate and other weak acids (Greenwald, Redish & Kibrick, 1940). In a study on the interaction between sodium triphosphate and $MgCl_2$ it was found by the author that the pH of the solution decreased gradually as the $MgCl_2$ concentration was raised (Frankenthal, 1944). Rogers & Reynolds (1949) made a similar observation on titrating solutions of $Na_4P_2O_7$ with a $MgCl_2$ solution. In view of this effect it was considered desirable to determine the dependence of pH on the concentration of $MgCl_2$ present in a solution of constant $Na_4P_2O_7$ concentration.

Effect of $MgCl_2$ *concentration on the* pH *of pyrophosphate solution*

Equal volumes of a freshly prepared $Na_4P_2O_7$ solution were added to $MgCl_2$ solutions of varying concentration, the final concentration of $Na_4P_2O_7$ being in each case $1{\cdot}345 \times 10^{-3}$ M. The pH of each mixture was determined immediately after the addition of $Na_4P_2O_7$ and the values obtained were plotted against the $MgCl_2$ concentration (Fig. 1). As may be seen from the curve in Fig. 1, the pH depression brought about by a $MgCl_2$ concentration of $0{\cdot}513 \times 10^{-3}$ M is slight, amounting to 0·1 pH unit. However, the depressing effect increases considerably as $MgCl_2$ concentration is raised, causing a pronounced increase in the slope of the curve towards an almost vertical position which continues until pH 8·13, corresponding to a $MgCl_2$ concentration of $2{\cdot}05 \times 10^{-3}$ M, is reached. At this point a second pronounced change in slope, this

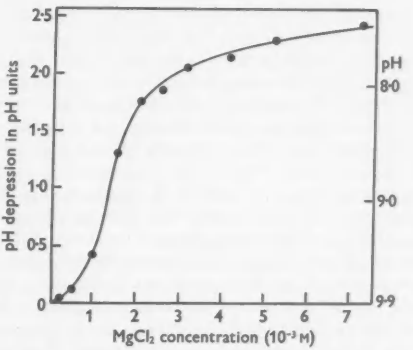

Fig. 1. The effect of the concentration of added $MgCl_2$ on the pH of a $1{\cdot}345 \times 10^{-3}$ M solution of $Na_4P_2O_7$.

time to a nearly horizontal position, occurs, and further additions of $MgCl_2$ cause relatively small pH depressions, as may be seen from the last part of the curve. A similarly shaped curve was obtained on repeating this experiment in the presence of a solution of erythrocyte pyrophosphatase.

The effect on the pH caused by the addition of $MgCl_2$ to the pyrophosphate solution was taken into consideration when adjusting the pH for enzyme experiments. Although these experiments were carried out in the presence of buffer, every precaution was taken to adjust the reaction mixture to pH 7 before the addition of same.

Effect of $MgCl_2$ *concentration on the rate of enzymic hydrolysis of pyrophosphate*

The experiment designed to study the effect of $MgCl_2$ concentration on the rate of hydrolysis of pyrophosphate, catalysed by purified erythrocyte pyrophosphatase, was carried out with the same concentrations of $MgCl_2$ and pyrophosphate as the previous one. Hydrochloric acid was added to the mixtures as required to adjust the pH to 7–7·2. Veronal/acetate buffer (Michaelis, 1931) of pH 7 was also added. The same order of addition of ingredients was followed in all the enzymic experiments.

Pyrophosphate was always added immediately before the enzyme, which was put in last. This was done in order to avoid the ageing of the $MgCl_2$/pyrophosphate mixtures before their coming into contact with the enzyme. The enzyme concentration amounted to 0·2 ml. eluate/10 ml. digestion mixture. The digestion mixtures were incubated at 37° and at given time intervals their phosphate content was determined (the pH was always determined at the beginning of incubation). The results obtained after 30 min. incubation are plotted against $MgCl_2$ concentration in Fig. 2. As may be seen from the curve, the rate of enzymic hydrolysis of pyrophosphate increases gradually as the $MgCl_2$ content of the reaction mixture is raised. It is significant that the curve follows the same course as the one obtained for the pH depression against $MgCl_2$ concentration. Both curves show sharp changes of slope at the $MgCl_2$ concentrations of $0{\cdot}5 \times 10^{-3}$ M and $2{\cdot}1 \times 10^{-3}$ M (corresponding to $Mg^{2+}/P_2O_7^{4-}$ ratios of 0·4 and 1·6 approximately), the effect of $MgCl_2$ being most pronounced between these two points. By raising the $MgCl_2$ concentration above $3{\cdot}1 \times 10^{-3}$ M, further, but small, increases in the hydrolysis rate and pH depression are brought about, as may be seen from the nearly horizontal branches of the curves. Similar curves were obtained in experiments with different enzyme concentrations. This is in accordance with the finding of Kunitz (1952), who reported that the concentration of magnesium required to bring about the optimum activation of crystalline pyrophosphatase does not depend on the concentration of the enzyme used in a concentration of about 0·2 μg./ml. digestion mixture.

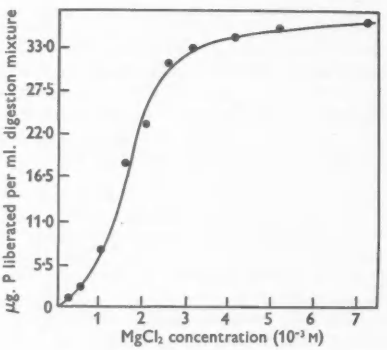

Fig. 2. The effect of $MgCl_2$ concentration on the rate of hydrolysis of pyrophosphate by erythrocyte pyrophosphatase at pH 7·1. Concentration of enzyme, 0·2 ml. eluate/10 ml. digestion mixture; concentration of pyrophosphate, $1{\cdot}345 \times 10^{-3}$ M; concentration of buffer, $1{\cdot}43 \times 10^{-2}$ M; incubation time; 30 min.

Effect of enzyme concentration and pH *on the rate of hydrolysis of pyrophosphate*

The concentration of $Na_4P_2O_7$ was $1{\cdot}345 \times 10^{-3}$ M. The concentration of $MgCl_2$ chosen in these and in all further experiments in which optimum activation was desired was $5{\cdot}13 \times 10^{-3}$ M. Under these conditions, where the $Mg^{2+}/P_2O_7^{4-}$ ratio of 3·81 is reached, a nearly optimum activation is achieved (as may be seen from the horizontal part of the curve in Fig. 2) without any precipitation occurring for an hour at least (Table 1). The results obtained with erythrocyte pyrophosphatase are in agreement with previous findings for pyrophosphatases from other sources (Bailey & Webb, 1944; Nagana & Menon, 1948; Gordon, 1950; Kunitz, 1952) and show that the enzymic hydrolysis of pyrophosphate is optimum at pH 7·1 and directly proportional to enzyme concentration.

Effect of initial substrate concentration on the hydrolysis of pyrophosphate

An increase of $Na_4P_2O_7$ concentration in the digestion mixture at pH 7, accelerated the rate of its enzymic hydrolysis as long as $MgCl_2$ was present in excess (see also Kunitz, 1952; Gilmour & Calaby, 1953). When, however, $MgCl_2$ was present in approximately equimolar concentration or less in relation to pyrophosphate ($Mg^{2+}/P_2O_7^{4-} \not> 1$) the addition of more $Na_4P_2O_7$ inhibited the reaction (Table 2) (see also Bauer, 1937; Bailey & Webb, 1944; Heppel & Hilmoe, 1951; Gilmour & Calaby, 1953; McElroy, Coulombre & Hays, 1951). Heppel & Hilmoe (1951) attribute the inhibition caused by high concentrations of pyrophosphate to its binding magnesium ions and withholding them from the active centres of the enzyme.

In a second experiment, designed to study the effect of initial substrate concentration (also carried out at pH 7), the $MgCl_2$ concentration was varied together with that of pyrophosphate, in order to maintain a nearly optimum activation at the constant $Mg^{2+}/P_2O_7^{4-}$ ratio 3·81. The results obtained show a gradual increase in the rate of

Table 2. *The effect of increasing pyrophosphate concentration on the rate of its enzymic hydrolysis in the presence of high and low* $MgCl_2$ *concentrations at* pH 7·1

Concentration of buffer: $2{\cdot}86 \times 10^{-2}$ M; concentration of eluate: 0·2 ml./10 ml. digestion mixture; incubation time: 30 min.

$MgCl_2$ concentration (10^{-3} M)	$Na_4P_2O_7$ concentration (10^{-3} M)	μg. P liberated/ml. digestion mixture
2·57	0·673	26·7
2·57	1·345	32·2
0·77	0·673	13·5
0·77	1·345	10·3

hydrolysis as the initial substrate concentration is raised. Fig. 3 shows the plot of $1/v_0$ against $1/S_0$ in accordance with linear equation of Lineweaver & Burk (1934):

$$\frac{1}{v_0}=\frac{Km}{V_0}\frac{1}{S_0}+\frac{1}{V_0}.$$

This equation is the reciprocal of the Michaelis-Menten equation (1913):

$$v_0=\frac{V_0S_0}{Km+S_0};$$

v_0 = initial rate of hydrolysis; V_0 = maximum rate of hydrolysis; S_0 = initial concentration of substrate; Km = 'Michaelis constant'.

The plotted points fall on an essentially straight line. It is thus evident that the data obtained fit the Michaelis-Menten equation. The calculated 'Michaelis constant' derived from these data is $Km = 5{\cdot}4 \times 10^{-4}$.

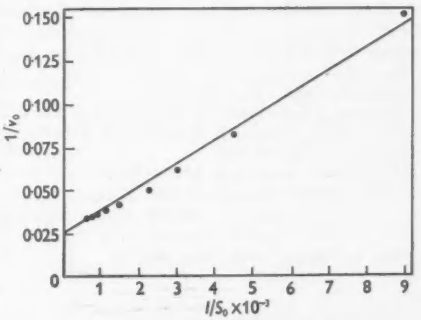

Fig. 3. The effect of initial concentration of sodium pyrophosphate (at constant $Mg^{2+}/P_2O_7^{4-}$ ratio and optimum activation) on the rate of its hydrolysis by erythrocyte pyrophosphatase at pH 7·1. v_0 = μg. P liberated/ml. digestion mixture. S_0 = initial concentration of pyrophosphate which varied between $0{\cdot}112 \times 10^{-3}$ and $1{\cdot}568 \times 10^{-3}$ M. Concentration of enzyme, 0·2 ml. eluate/10 ml. digestion mixture; concentration of buffer, $1{\cdot}43 \times 10^{-2}$ M; incubation time, 15 min.; ratio of $Mg^{2+}/P_2O_7^{4-} = 3{\cdot}81$.

DISCUSSION

The results suggest that the actual substrate of erythrocyte pyrophosphatase (possibly also of other inorganic pyrophosphatases) is not $Na_4P_2O_7$ or $HP_2O_7^{3-}$ (according to Bailey & Webb's formulation, 1944), but the complex ion $(MgP_2O_7)^{2-}$ contained in the solution of $MgCl_2$ + pyrophosphate. The reaction of complex formation might be formulated as follows:

$$Mg^{2+} + (HP_2O_7)^{3-} \rightleftharpoons H^+(MgP_2O_7)^{2-}.$$

Accordingly, high $MgCl_2$ concentrations will further the forward reaction in the direction of complex (or substrate) formation, and the more magnesium there is in the reaction mixture, the more substrate there will be (as long as no precipitation occurs), and the higher the rate of hydrolysis (Fig. 2).

It is evident from the above formulation that at constant $Mg^{2+}/P_2O_7^{4-}$ ratio, the concentration of the complex ion will be directly proportional, and in the presence of a high excess of Mg^{2+} practically equal, to that of pyrophosphate concentration. The data obtained in the experiment on 'the effect of initial substrate concentration on the hydrolysis of pyrophosphate' in which a nearly optimum $MgCl_2$ concentration at a constant $Mg^{2+}/P_2O_7^{4-}$ ratio was maintained—could thus be expected to fit the Michaelis-Menten (1913) equation, as indeed was the case. It should be pointed out that for the calculation of Km the initial concentration of pyrophosphate was introduced for S_0 in the Michaelis-Menten equation assuming it to be approximately equal to that of the actual substrate, under the conditions of the experiment.

Since the complex formation is accompanied by a decrease in the pH of the mixture, one would expect a reversal of the complex-forming reaction on addition of H^+ ions. This explains the very high ratios of $MgCl_2$ to $Na_4P_2O_7$ found necessary for optimum activity of pyrophosphatase at pH 5·3 (Bauer, 1937; Kunitz, 1952). The higher $Mg^{2+}/P_2O_7^{4-}$ ratio necessary in the case of high dilutions can be explained in the same way.

Since the pH depression resulting from the addition of $MgCl_2$ to a $Na_4P_2O_7$ solution is in a way a measure of complex formation, it is not surprising that the hydrolysis rate of this complex follows the same course as the pH depression when plotted against $MgCl_2$ concentration. One would expect, however, that increasing the pyrophosphate concentration in the digestion mixture would also lead to an acceleration of the rate of hydrolysis by furthering the complex formation, just as increasing the $MgCl_2$ concentration does. This is indeed the case in the presence of an excess of $MgCl_2$ (Table 2). The inhibition caused by an increase of pyrophosphate concentration in the absence of an excess of $MgCl_2$ can be explained on the assumption that pyrophosphate as such can combine with the enzyme, but that no hydrolysis results from this combination (Bauer, 1937). An excess of pyrophosphate, while slightly forcing increased complex formation, at the same time competes for the enzyme with the true substrate $(MgP_2O_7)^{2-}$. This blocks the enzyme and inhibits hydrolysis.

The suggestion that the complex ion $(MgP_2O_7)^{2-}$ plays the role of the actual substrate for inorganic pyrophosphatase might also explain the fact that organic derivatives of pyrophosphate, such as ATP, adenosine diphosphate, thiamine pyrophosphate, etc., are not hydrolysed by this enzyme (Kunitz, 1952; McElroy *et al.* 1951).

SUMMARY

1. The preparation of an eluate of erythrocyte pyrophosphatase from rat blood is described.

2. This enzyme requires the presence of magnesium to catalyse the hydrolysis of inorganic pyrophosphate into orthophosphate ions. It does not catalyse the hydrolysis of glycerophosphate or thiamine pyrophosphate under these conditions.

3. The rate of the enzymic pyrophosphate hydrolysis is a function of the concentration of magnesium chloride.

4. There is a similarity between the plot of the rate of enzymic hydrolysis against magnesium chloride concentration, and of pH depression against magnesium chloride concentration in the pyrophosphate solution.

5. The rate of the enzymic hydrolysis of pyrophosphate is directly proportional to enzyme concentration and is a function of pH. The activity is optimum at pH 7·1.

6. The plot of the initial rate of hydrolysis against initial pyrophosphate concentration fits the Michaelis-Menten equation when magnesium chloride concentration is varied with that of the substrate to maintain a constant and nearly optimum $Mg^{2+}/P_2O_7^{4-}$ ratio. *Km* was found to be equal to $5{\cdot}4 \times 10^{-4}$.

7. It is suggested that the actual substrate for inorganic pyrophosphatase is neither $Na_4P_2O_7$ nor $(HP_2O_7)^{3-}$ but the complex anion $(MgP_2O_7)^{2-}$.

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Actinomycete Metabolism: α-Phenylmannosidase of *Streptomyces griseus*

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Langlykke & Perlman (1950) have reported that *Streptomyces griseus* forms an enzyme capable of converting mannosidostreptomycin into the aglycone, streptomycin, and have described the dependence of its activity upon oxidation potential and pH value and its inhibition by heavy metals. During the course of our work it was found that synthetic phenyl α-D-mannoside was also split by the organism. Accordingly we have used this synthetic substrate to provide such general information on α-mannosidase activity as might later be used in work on the natural substrate. Although in this work the term 'mannosidase', which is used for convenience, implies a simple hydrolysis of mannosidostreptomycin or α-phenylmannoside into mannose and streptomycin or phenol, we do not exclude the possibility that the enzymic cleavage of either substrate is achieved by a more complex mechanism.

METHODS AND MATERIALS

Organism. Streptomyces griseus mutant (Dulaney, Z38) was maintained and grown on soya bean medium, as in example I of the patent describing it (Dulaney, 1951). Growth was always carried out at 28·5° on a rotary shaker (160 rev./min.) in quantities of 40 ml. medium in 250 ml. conical flasks.

Determination of enzyme activity. The substrate was phenyl α-D-mannoside, prepared by the method of Helferich & Winkler (1933) with the modification that deacetylation was carried out as by Zemplen & Kunz (1923). The determinations were based on measurement of the amount of phenol liberated.

In our early experiments we removed the phenol by distillation, using a method similar to that described by Volterra (1942) but the pH was adjusted to 6·4 with 0·1 M-KH_2PO_4, and CO_2 was also bubbled through the boiling liquid to prevent frothing. The phenol content of the distillate was determined colorimetrically (Folin & Ciocalteu, 1927). This method not only proved tedious, but also needed large samples, and simpler ones were tried. Extraction with chlorinated solvents (cf. Ellinger, Ruchhoft & Lishka, 1951) was difficult on account of persistent emulsion formation. A search was then made for reagents for estimating phenol which were not subject to interference by broth constituents. The procedure of Gottlieb & Marsh (1946), based on colour development with 4-aminoantipyrin, was found suitable in sensitivity, specificity and stability of colour formed. It was found convenient to clear the samples by addition of ethanol, which did not interfere with the test. Other methods of colorimetric estimation were rejected, that of Folin & Ciocalteu (1927) because of inconsistent blank values, that with diazotized sulphanilic acid (Schmidt, 1949) because of the instability of the colour and that involving the use of halogenated quinone chlorimides to form coloured indophenols (Singer & Stern, 1951) because of the slow rate of attaining maximum colour.

Enzyme activity was defined as being unity when 1 μm-mole of phenol is liberated per ml. in 30 min. at the temperature of incubation. The activity varies with temperature and unless otherwise stated all determinations recorded here were carried out at 28·5°. The enzyme is sensitive to aeration and therefore a standard set of conditions is laid down for the operation of the test, as below. The reagents used were: *A*, glycine buffer, pH 10·4, prepared by mixing 54 vol. of an aqueous solution containing 4·51 g. glycine and 3·48 g. NaCl in 600 ml. and 46 vol. of 0·1 N-NaOH; this is adjusted to pH 10·4 with the appropriate constituent solutions; *B*, 1% (w/v) aqueous 4-aminoantipyrin; *C*, 3% (w/v) aqueous potassium ferricyanide A.R.; *D*, 1·0% (w/v) aqueous α-phenylmannoside.

Incubation unit. A 6 × 1 in. boiling tube with a 'B24' socket to fit a gas wash bottle head (MF 28/3, Quickfit and Quartz, Ltd. London), whose inlet tube reaches to within 8 mm. of the bottom was immersed in a temperature-controlled water bath and connected in series to other units and an air supply delivering 700–900 ml./min. A vibrator-type aquarium aerator was found most suitable for a constant-rate air supply.

Enzyme activity. 15 ml. of enzyme solution (e.g. culture fluid) were transferred into the incubation unit, which was allowed to equilibrate to the required temperature for 5 min. To this was added 1 ml. of *D*; the aeration train was then connected and incubation was carried out for exactly 30 min. Immediately after this, 14 ml. of 96% ethanol (industrial methylated spirits) were added with good mixing and the solution was then filtered through a Whatman no. 42 fluted filter.

To 1 ml. of filtrate were added 10 ml. *A*, 1 ml. *B* and 3 ml. *C*, with good mixing. The colour developed was measured in a Spekker Absorptiometer (Ilford no. 605 filters; $\lambda_{max.}$, 545 mμ.) against an enzyme blank consisting of 1 ml. of filtrate from a mixture of 15 ml. of enzyme solution and 15 ml. of 96% ethanol. The readings must be taken within 10–40 min. after the reagent additions.

Standard calibration graphs were constructed by adding 1 ml. *B* and 3 ml. *C* to the following mixtures:

glycine buffer *A* (ml.)	10	9	8	7	6
5×10^{-5} M phenol (ml.)	1	2	3	4	5
to give units of enzyme activity	100	200	300	400	500

The developed colours were read against a water blank. The phenol solutions were checked by bromimetric or iodimetric analysis.

The method was checked to show the reproducibility of the phenol assay by determination of added phenol (Table 1), and the reproducibility of enzyme activity for the same broth samples (Table 2). Agreement was found to be excellent. We also investigated the effect of storage at $-10°$ on the enzyme in whole cultures and found that after 4 days no deterioration in activity had occurred.

It has also been found that the requisite aeration may be obtained by shaking 15 ml. of enzyme preparation in a 250 ml. conical flask on a rotary shaker, as described above, at 28·5°. This method was used for many of the determinations quoted below, as it was useful for carrying out a number of investigations at the same time, when comparative rather than absolute values were important. The units of activity, which differ little from those obtained in tubes (units (T)/ml.), will be given as units (S)/ml.

Whole cultures were used as enzyme sources. Very little work has yet been carried out on the purification of the enzyme.

RESULTS

Effect of time and substrate concentration

Under the conditions of aeration, temperature and pH described above, the amount of phenol liberated by the enzyme increased linearly with time (Fig. 1). In experiments in which enzyme action was allowed to continue beyond a point at which the amount of liberated phenol was about 200 μg./ml., some slowing down was observed. This was first thought to be due to an inhibitory effect of phenol, but was found not to be so, as the addition of phenol (200 μg./ml.) caused no retardation of enzyme activity. On the other hand, mannose (380 μg./ml.) did cause some inhibition.

For accurate enzyme estimations it is necessary to work on the portion of the time curve that is linear, i.e. up to about 2 μmole of liberated phenol/ml. broth.

Determination of the Michaelis constant

Though the Michaelis constant for a pure enzyme and the substrate concentration at which the enzyme shows one half its maximum activity in culture broth are not necessarily identical, the constant is still of some use in characterizing the enzyme. A number of determinations were made with 10 ml. portions of broth to which was added 1 ml. of aqueous substrate solution containing sufficient α-phenylmannoside to give concentrations of 136, 55, 27 and 11 μg./ml. of incubation mixture. Incubation with shaking was carried out for 10 min. and the liberated phenol estimated in the usual way. A typical set of figures is shown in Table 3.

In the range, 136–55 μg./ml., the liberation of phenol was, within experimental error, the same. Below this level, the hydrolysis of α-phenylmannoside was complete at the end of the incubation

Table 1. *Recovery of added phenol from non-incubated whole cultures*

For details of the estimation of phenol see text. A and B represent duplicate determinations on the same fortified broth culture sample; a and b represent duplicate colorimetric determinations on the same ethanolic filtrates.

Sample	Added phenol (μg./ml.)	Phenol recovered (μg./ml.)		Recovery (%)	
		a	*b*	*a*	*b*
1*A*	16·9	17·9	17·5	105·5	103·5
1*B*	16·9	16·7	16·7	98·8	98·8
2*A*	33·1	32·1	32·5	97·1	98·2
2*B*	33·1	32·9	32·9	99·4	99·4
3*A*	53·7	52·5	53·3	97·8	99·3
3*B*	53·7	54·2	53·8	101·0	100·1
4*A*	63·7	61·9	61·9	97·2	97·2
4*B*	63·7	63·7	63·7	100·0	100·0
		Average recovery		99·6	99·6

Table 2. *Reproducibility of enzyme activity determinations under standard conditions*

For details of the estimation and definition of enzyme activity see text. Standard deviation of individual determination, 1·4%.

Culture sample	Enzyme activity (units (T)/ml.)		
A	294	289	289
B	187	188	195
C	564	552	554
D	952	962	953
E	277	276	276
F	366	365	365

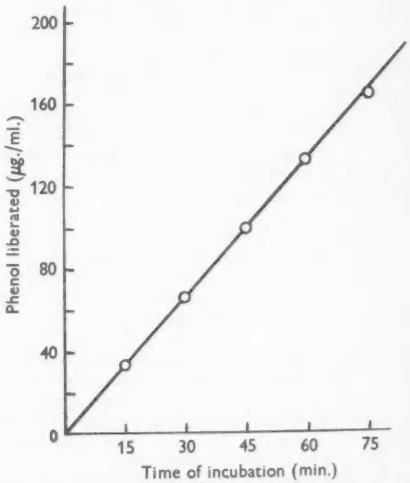

Fig. 1. Relationship between time of incubation and liberation of phenol from α-phenylmannoside by whole cultures of *Streptomyces griseus*. For conditions see text.

Table 3. *The effect of substrate concentration on enzyme activity*

10 ml. portions of culture broth were incubated with varying amounts of α-phenylmannoside in 1 ml. water. For further details see text.

Substrate concentration (μg./ml.)	Phenol liberated (μg./ml.) In experiment	Phenol liberated (μg./ml.) Theoretically possible
136	14·2	50·0
55	13·7	20·0
27	10·3	10·0
11	4·4	4·0

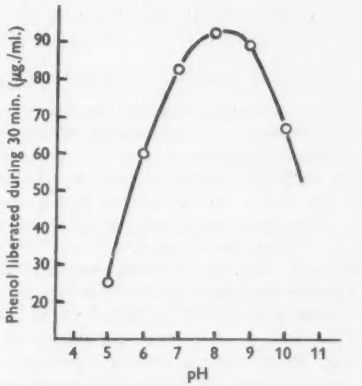

Fig. 2. The effect of pH on the hydrolysis of α-phenylmannoside by whole cultures of *Streptomyces griseus*. For conditions see text.

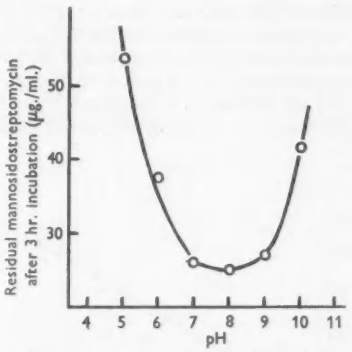

Fig. 3. The effect of pH on the hydrolysis of mannosidostreptomycin. For conditions see text.

period and the liberated phenol gives no indication of the reaction velocities. In order to measure the initial velocities, shorter incubation periods would have to be chosen in such a way that the liberated phenol could be measured before total hydrolysis occurs, but the amount of liberated phenol would then be too small for accurate estimation. All that can be deduced from the present experiments is that a Michaelis constant in excess of 10 μg./ml., i.e. approx. 4×10^{-5} M, would not be consistent with the experimental findings.

Effect of hydrogen-ion concentration

Samples of a standard 6-day culture were adjusted to various pH values with N-HCl or N-NaOH and brought to equal volumes by small compensating additions of water. The activity (units (*S*)/ml.) was determined for each under otherwise standard conditions. Fig. 2 indicates that optimum activity was shown at about pH 8·0, but that there was less than a 10 % fall from this value at either pH 7·0 or 9·0. This showed that, as active cultures without exception had pH values between 7·0 and 9·0, there was only need to adjust the pH value of the enzyme system in the most exacting experiments. As a rule, therefore, this was not done. Beyond these limits activity declined rapidly to 30 % of maximum at pH 5·0 and 70 % of maximum at pH 10·0.

A similar experiment with mannosidostreptomycin as substrate (Fig. 3) indicated that the optimum of the culture for cleavage of this substrate was again about pH 8. In this experiment 15 ml. portions of culture broth (containing a negligible amount of mannosidostreptomycin) were adjusted to various pH levels as in the above experiment. Solid mannosidostreptomycin hydrochloride was added to give a concentration of 100 μg./ml. The mixtures were incubated with shaking for 3 hr. (trial experiments had shown that the cleavage of mannosidostreptomycin was much slower than that of α-phenylmannoside). Residual mannosidostreptomycin was then estimated by the method of Emery & Walker (1949).

Effect of aeration upon enzyme activity

Enzyme—substrate mixtures were made as for the standard shake-flask test, but were distributed in various quantities in smaller (100 ml.) conical flasks, in order to vary the degree of aeration. Incubation with shaking of 15, 45 and 75 ml. portions of digest for 15 min. gave enzyme activities of 740, 600 and 220 units (*S*)/ml., respectively.

A more precise experiment was carried out under the conditions of the tube test, which was set up in the standard way except that the rate of passage of air was varied (Fig. 4). It is clear that the rate of aeration must be fixed; for the standard test, as mentioned above, it was set at 700–800 ml./min. The apparent decrease in enzyme activity with very high aeration is due to loss of phenol from the incubation unit, as was proved by passing the exit air through alkali and detecting phenol in the alkali.

Inhibition by anaerobiosis was reversible, shown by incubating broth anaerobically in presence of substrate and subsequently under aerobic conditions. Three 10 ml. portions of an enzyme-substrate digest were treated in the following way. The first was incubated anaerobically for 30 min., the second aerobically for 30 min. and the third portion aerobically and then anaerobically for the same length of time; 12, 49 and 56 μg./ml. phenol were liberated in the respective digests. This experiment was repeated and the same effect obtained after 16 hr. of anaerobiosis.

A similar dependence on aeration has been found when mannosidostreptomycin was used as substrate. In a typical experiment 20, 30, 40 and 50 ml. of culture broth to which 620 μg./ml. mannosidostreptomycin had been added were placed into 50 ml. conical flasks, which were then plugged with cotton wool. A fifth flask was filled completely with a similar mixture and closed with a rubber bung. Mannosidostreptomycin was determined after incubating with shaking for 3 hr.; 90, 85, 100, 320 and 550 μg./ml. of mannosidostreptomycin were found in the respective flasks.

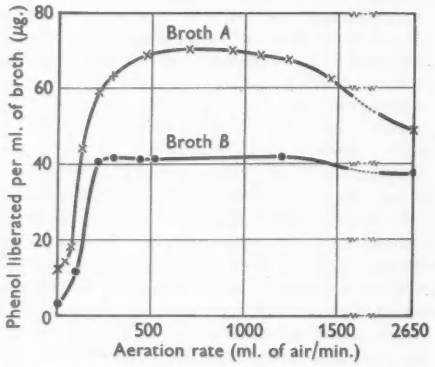

Fig. 4. Showing the effect of aeration on the α-phenylmannosidase activities of two broths (*A* and *B*) of *Streptomyces griseus*. For conditions see text.

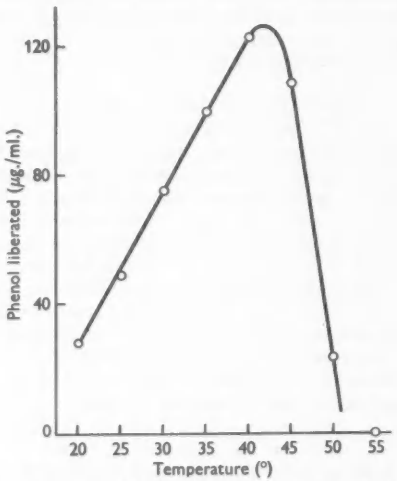

Fig. 5. Effect of temperature on the hydrolysis of α-phenylmannoside. For conditions see text.

Effect of temperature upon enzyme action

Under standard shaking conditions the optimum temperature for enzyme action was found to be about 40°. The enzyme appears to be very heat labile and is completely inactivated at 55° (Fig. 5).

The effect of phosphate on enzyme activity

In order to decide whether the cleavage of α-phenylmannoside was hydrolytic or phosphorolytic, incubation was carried out on the shaker in the presence of 0·02 M sodium phosphate of the same pH as the broth. To the control broth the same amount of sodium (as sodium chloride) was added, to eliminate any effect due to sodium. The results are shown in Fig. 6. A similar experiment with 0·05 M phosphate again showed that phosphate did not increase the rate of hydrolysis; it is therefore unlikely that α-mannoside is split phosphorolytically. (The concentration of inorganic phosphate of a normal 6-day old culture filtrate at the time of determination of enzyme activity was about 0·0003 M.)

Effect of inhibitors

Inhibition due to sugars. A number of sugars were added to enzyme preparations at 0·05 M concentration and their effect on the liberation of phenol from α-phenylmannoside over a period of 30 min. (with shaking) was ascertained. The results from a number of experiments on different broths are shown in Table 4.

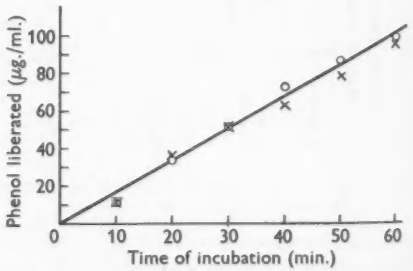

Fig. 6. Effect of added phosphate on rate of hydrolysis of α-phenylmannoside. ×—×, Added 0·02 M phosphate; ○—○, control. For conditions see text.

Effect of metal ions. The enzyme activity was reduced only by relatively high concentrations of Cu^{2+} and Fe^{2+} (as sulphates). In a number of culture broths these metals at a concentration of 0·001 M had no effect on enzyme activity, whereas a concentration of 0·01 M invariably produced strong enzyme inhibition. In some experiments, however, there was also some inhibition with 0·001 M metal salts. K^+, Mg^{2+}, Ca^{2+} and Ba^{2+} (all 0·01 M) had no effect on enzyme activity. The enzyme activities in these experiments were determined after incubation on the shaker.

Effect of sulphite, iodoacetate, arsenate and cyanide. Solutions of the sodium salts of these substances, adjusted to the pH of the culture broth with sodium hydroxide or hydrochloric acid, were added to give a concentration of 0·01 M. Incubation with shaking for 30 min. in the presence of substrate showed that sulphite and cyanide caused 62 and 90 % inhibition, respectively, arsenate stimulated enzyme activity by 40 % and iodoacetate had no effect. (These figures represent average values from several experiments.)

Table 4. *Effect of carbohydrates on enzyme activity*

Duplicate figures for the inhibition by individual sugars were obtained from separate digests, not from samples of the same digest. For other details see text.

Sugar (0·05 M)	Approx. % inhibition
Glucose	5, 2, 7
Fructose	7, 7, 0
D-Arabinose	7, 13
D-Ribose	2, 6
D-Xylose	11
Rhamnose	0, 0
Sucrose	13, 3, 2, 12
Mannitol	4, 0
α-Methylglucoside	9, 20, 12
Maltose	66, 81
Cellobiose	80, 84
Mannose	87, 87, 80, 79
α-Methylmannoside	98, 93, 96, 96

Correlation of mannosidostreptomycin level with appearance of phenyl α-D-mannosidase activity

In a number of experimental cultures the amount of mannosidostreptomycin was determined by the method of Emery & Walker (1949) and compared with the α-mannosidase activity over a number of days. It was found that high mannosidase activity was paralleled by a low level of mannosidostreptomycin. When there was no fall in mannosidostreptomycin level, little or no enzyme activity was manifest (Table 5).

DISCUSSION

The sensitivity of the enzyme to aeration conditions is noteworthy. A similar phenomenon for hydrolytic enzymes was described recently (Yamagita & Nishi, 1952), when it was shown that proteolytic hydrolysis by trypsin or chymotrypsin and the autocatalytic conversion of trypsinogen into trypsin were inhibited under anaerobic conditions. Since cell lysis starts soon after the disappearance of glucose from the medium and is followed by the appearance of α-mannosidase activity, it would appear possible that the enzyme might be intracellularly present earlier in the fermentation, and that lysis might activate it by bringing it into contact with a higher oxygen tension.

The affinity of the enzyme for α-phenylmannoside was found to be higher than for its natural substrate mannosidostreptomycin. The Michaelis constant for the latter has been found in our laboratories to be $1{\cdot}72 \times 10^{-3}$ M (P. B. Dickenson & J. S. Hughes, personal communication). Owing to the difficulty of determining phenol at the necessary low levels, we were not able to estimate the Michaelis constant for the synthetic substrate. It seems, however, to be appreciably lower than that found for the natural substrate. Moreover, the rate of hydrolysis of mannosidostreptomycin was only

Table 5. *Relation between mannosidostreptomycin content and α-phenylmannosidase activity of cultures*

% M, mannosidostreptomycin as molar % of total streptomycin. *E*, α-phenylmannosidase activity (units (S)/ml.; in *A*, *B*, *C*, *D* and units (T)/ml.; in *E*, *F*, *G*, *H*, *I*).

	Duration of fermentation									
	Day 3		Day 4		Day 5		Day 6		Day 7	
Culture sample	% *M*	*E*	% *M*	*E*	% *M*	*E*	% *M*	*E*	% *M*	*E*
A	—	—	46	0	50	0	60	40	—	0
B	—	—	39	0	8	1490	6	940	5	600
C	51	0	49	0	46	54	36	20	—	—
D	34	0	41	0	5	1930	8	1750	6	1120
E	—	—	—	—	33	0	32	0	29	0
F	31	0	33	35	43	45	29	175	—	—
G	31	0	30	35	25	205	3	995	—	—
H	56	0	32	245	8	1605	7	1235	—	—
I	40	0	43	0	19	905	7	340	—	—

about one-sixth of that of α-phenylmannoside. A similar finding was made by Cohn & Monod (1951), who showed that the affinity of an *Escherichia coli* β-galactosidase for synthetic *o*-nitrophenyl β-galactoside was greater than for lactose.

When breakdown to streptomycin occurs, the pH of a culture broth is usually just on the alkaline side, i.e. at the optimum pH for the action of the enzyme on either substrate. This optimum is in marked contrast to the optimum pH for the action of the α-mannosidases of almond emulsin and lucerne seeds (pH 3·3–5·5; Hill, 1934) or coffee emulsin (pH 4·5–5·5; Helferich & Vorsatz, 1935).

In view of the lack of effect of inorganic phosphate on the rate of hydrolysis of phenyl α-D-mannoside, which implies non-phosphorolytic breakdown, it is strange that arsenate should so markedly increase the reaction velocity.

Only four of the sugars tested, mannose, α-methylmannoside, maltose and cellobiose, were inhibitory. The first two probably acted as competitive inhibitors. It is not clear why the last two, an α-glucoside and a β-glucoside, should inhibit. It is possible that an irreversible enzyme-glucoside complex is formed. α-Phenylglucoside was tried as an enzyme substrate but was not hydrolysed.

The inhibition of the enzyme by the heavy metals copper and iron was presumably of a general nature. Although some changes in the activity of similar microbial glucosidases have been reported with K^+, Ca^{2+}, Mg^{2+} and Ba^{2+}, such an effect was not demonstrated on our enzyme. The failure to observe it may have been due to the use of a cell suspension instead of a purified enzyme.

Of general inhibitors, only sulphite, cyanide and iodoacetate were examined. The two former inhibited strongly, whereas iodoacetate had no effect. It appears, therefore, that the enzymic action of mannosidostreptomycinase is unlikely to depend on the presence of SH groups.

The late appearance in normal cultures of an enzyme releasing mannose from mannosidostreptomycin (mannose, as the phenylhydrazone, was isolated from such cultures by Perlman & Langlykke, 1948) makes it likely that the latter is a carbohydrate storage material for the organism (cf. Frey-Wyssling, 1942) and that release of carbohydrate is brought about when the glucose is depleted. The action of the mould on phenyl α-D-mannoside parallels this and would tend to support the hypothesis that cleavage of both substrates is effected by the same enzyme. The similarity of the pH activity curves found with the two substrates, the dependence of enzyme action for each substrate on aeration and the close correlation between mannosidostreptomycin level and α-mannosidase activity all suggest such an identity.

SUMMARY

1. It has been demonstrated that cultures of *Streptomyces griseus* possess an enzyme capable of liberating phenol from phenyl α-D-mannoside.

2. The optimum pH for the activity of the enzyme is approximately 8·0.

3. The enzyme is very sensitive to conditions of aeration. Decreased aeration causes a marked decrease of enzyme activity.

4. Mannose, methyl α-D-mannoside, maltose and cellobiose strongly inhibit the enzyme. A number of other sugars have little or no effect.

5. Ferrous and cupric ions have some inhibitory effect on the enzyme. K^+, Ca^{2+}, Mg^{2+} and Ba^{2+} have no effect.

6. The rate of enzymic cleavage of substrate is not influenced by inorganic phosphate. Arsenate increases the rate.

7. Cyanide and sulphite inhibit the enzyme, iodoacetate has no effect.

8. The Michaelis constant of the enzyme with respect to phenyl α-D-mannoside is less than 10^{-4} M.

9. It is probable that the same enzyme is responsible for the conversion of mannosidostreptomycin into streptomycin and the hydrolysis of phenyl α-D-mannoside.

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Molecular Size, Shape and Aggregation of Soluble Feather Keratin

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The keratins are epidermal proteins which contain a high percentage of cystine and, in the solid state, can form macroscopic structures in which there is a high degree of order in the arrangement of the polypeptide chains. Because of the last characteristic they have been an invaluable material to study the structure of proteins by the diffraction, or scatter, of X-rays. It was considered that measurements of the size and shape of keratin in solution would be of value in the interpretation of the X-ray diagrams, and the present work was undertaken to provide such data for feather keratin. To some extent the interaction between keratin particles in solution has also been considered since feather keratin is a suitable material for forming regenerated fibres (Lundgren, 1949), and a knowledge of the way keratin molecules can polymerize will be of value in devising ways of forming such fibres.

It has been known from the work of those interested in the mechanical properties of keratin fibres that the stability of the protein in the solid state is due to cross-linking by the cystine residues, to hydrogen bonds and to salt linkages (Lundgren, 1949). The addition of reagents which break these bonds decreases the strength of the fibres, and it is possible to dissolve keratin by mixtures of substances which break the —S—S— bonds and the hydrogen bonds, as was first shown by Goddard & Michaelis (1934), and later by Jones & Mecham (1943). The latter work is of particular value since solution was achieved under conditions that do not cause irreversible changes to the cystine residues.

In this study of the physical properties of feather-keratin solutions, the measurements of particle size and shape have been correlated with the cystine and cysteine content of the protein, the working hypothesis being that the keratin monomer would be a particle in which there is no cross-linking by —S—S— bonds.

Mercer & Oloffson (1951) and Ward (1952) observed the behaviour in the centrifugal field of wool keratin dissolved in solutions of urea and bisulphite but did not record the extent to which cross-linking by —S—S— bonds was present. Ward, High & Lundgren (1946) dissolved feather keratin in solutions of bisulphite and detergent and then removed the bisulphite by dialysis. The physical properties of the resultant solution were examined, but the cystine content of the dissolved protein was not recorded. It is known from the work of Middlebrook & Phillips (1942) that the reaction of wool keratin with bisulphite is partly reversible, and it has been found in the course of the present investigation that the removal of bisulphite from solutions of feather keratin in urea and bisulphite also results in loss of free —SH groups. It can therefore be presumed that the material studied by Ward *et al.* (1946) contained —S—S— bonds and was polymerized to an unknown extent.

MATERIALS

Starting material

White body feathers were obtained from Leghorn hens from the Hollywood Poultry Co. They were extracted with benzene at room temp. till the extract no longer gave a residue on distillation, and then dried in air and extracted again with distilled water (10 × 2 l./200 g. feathers), dried in air again, and kept *in vacuo* over P_2O_5 at room temp.

Extraction of soluble keratin

1 g. of feathers was suspended in 20 ml. of solvent and extracted at 40° for 20–24 hr., during which period any necessary adjustments to the pH of the extract were made. The suspension was filtered and the filtrate kept. The residue was washed with fresh solvent and then with distilled water till the washings were free from urea. The residue was then dried at 105° and weighed. Most extractions were made in 10M urea, 0·1M-$NaHSO_3$, 0·05M sodium phosphate buffer at various pH's. Some extractions were made in 10M urea, 0·5M sodium thioglycollate, 0·05M sodium phosphate buffer (pH 6).

When a solution in 5M urea, 0·1M bisulphite, 0·05M phosphate (pH 6–8·5) or in 5M urea, 0·5M thioglycollate, 0·05M phosphate (pH 6) was required, the extract made in 10M urea was diluted with an equal volume of 0·1M bisulphite, 0·05M phosphate buffer of the appropriate pH, or 0·5M thioglycollate, 0·05M phosphate buffer (pH 6), respectively. The dilution was made through a fine-bored pipette immersed in the solution, which was stirred during the dilution.

The protein in these solvents will be referred to as 'SH-keratin'.

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Treatment with performic acid (*cf. Sanger*, 1949)

Solutions of SH-keratin in 10M urea, 0·1M bisulphite, 0·05M phosphate buffer (pH 6–8·5) were dialysed against running tap water and then distilled water. An opalescent solution, or, when the protein concentration was above about 3%, a clear gel was produced, frozen, and dried by sublimation. 1 g. of this solid was suspended, and allowed to swell, in 30 ml. formic acid at room temp. After 30 min. 7 ml. H_2O_2 (30%, w/v) were added and the suspension was stirred. After about 15 min. a yellow solution was obtained and left at room temp. for a further 15 min. and then filtered through a sintered-glass filter. The filtrate was cooled to 5°, 5 vol. of acetone were added, and the resulting precipitate, which flocculated in about 30 min., was collected by centrifugation. The solid was washed with acetone, suspended in distilled water, frozen, and dried by sublimation. Yield, 95%.

This material will be known as 'cysteic acid-keratin'.

If the solution in the performic acid was diluted with water, incomplete precipitation occurred. Addition of acetone to the supernatant produced an opalescent solution from which the addition of NaCl (final concn. about 1M) produced complete precipitation.

METHODS

Nitrogen was determined by micro-Kjeldahl, using selenium catalyst and digesting for 8 hr.

Cystine and cysteine were determined by the methods of Shinohara (1935). The apparent cystine content of feather was the same when it was hydrolysed in 5N-HCl for 4, 6, 8, or 12 hr., and as a routine method the solid keratin was hydrolysed in 5N-HCl for 6 hr. under reflux. When the cysteine and cystine content of keratin in urea/bisulphite solution were required, the solution was pipetted into an equal volume of 10N-HCl which was vigorously stirred. The resultant solution was boiled under reflux for 6 hr. There was 95–102% recovery of cystine added to these solutions or to the feathers.

It is shown below that the relative apparent amount of cysteine and cystine in the soluble keratin dissolved in urea/bisulphite is dependent on the pH of the solution, the ratio of cystine to cysteine increasing as the pH is changed from 6 to 9. There is doubt whether the ratios found for these amino acids combined in keratin, dissolved in urea/bisulphite, at a certain pH, are indeed the true ones since by this analytical method there will be a large pH change on addition of the keratin solution to 10N-HCl. Cystine can be recovered quantitatively if it is added to 5N-HCl containing bisulphite but there may be a conversion of combined cystine into combined cysteine at the moment at which the keratin solution is added to the acid. However, this possibility will not affect the validity of the conclusions to be drawn from the cysteine/cystine ratios since these conclusions are based on the presence of cystine and the action of the acid would be to reduce that cystine.

Moisture contents were determined by drying to constant weight at 105°.

Ash was determined gravimetrically as the sulphate.

Determination of protein concentration. The concentration of keratin in solution can be determined from the refractive increment, the cystine content, or from the non-diffusible N content. The specific refractive increment of cysteic acid-keratin, dissolved in and dialysed against 0·05M-Na_2CO_3, 0·2M-NaCl was found to be 0·00183, the concentration being determined from the N content, which was taken as 16·5% for dry solid keratin. The value of the specific refractive increment of cysteic acid-keratin in 5M urea, 0·1M bisulphite, 0·05M phosphate was 0·00208, concentration being determined from the non-diffusible N content. It is presumed that this value applies to SH-keratin dissolved in this solvent.

In the determination of the non-diffusible N content, 1 ml. samples of the keratin solution were pipetted into cellophan sacs and dialysed against running tap water for 3 days. The keratin molecules are small enough to pass through the pores of the sac, and it was necessary to apply a correction for this loss. To that end a solution of cysteic acid-keratin was made in 0·05M-Na_2CO_3 and its N content determined. The solution was diluted with an equal volume of 10M-urea and samples of this placed in sacs and the non-diffusible N content determined. 10–12% of the keratin was lost after dialysis in this way for 3 days. The daily loss of keratin was constant, and it was therefore ascribed to the small size of the keratin molecule and not to a diffusible fraction.

Osmotic pressure measurements were made in toluene osmometers at 30°. Visking cellophan was used as the membrane (Visking Corp. Chicago, U.S.A.). About 5 ml. of protein solution and 125 ml. of solvent were used. The loss of cystine-containing material from the inside of the sac was 1%/day and as the corresponding decrease in the height of the column of toluene was small compared with the total height, this loss was neglected. The osmometers came to a steady state after 4 days. The ratio P/C was plotted against C and the values at zero concentration used to calculate the number-average molecular weight. (P = pressure in mm. Hg; C = concentration of keratin in g./100 ml. solution.)

Refractive indices were measured on a differential refractometer (Brice & Halwer, 1951), which was sensitive to a refractive index change of 0·000005. The temperature was 25° and the wavelength 546 mμ.

Viscosity measurements were made in Ostwald viscometers at 30°. The flow time for 4 ml. of distilled water was 277 sec. at 20°. Kinetic energy corrections were not made. The keratin solutions were filtered through ultrafine sintered-glass filters and then dialysed against the solvent. Before being placed in the viscometer they were centrifuged. Flow times were reproducible to 0·5 sec.

Ultracentrifuging was done in a Spinco Model E analytical centrifuge. The synthetic-boundary cell described by Pickels, Harrington & Schachman (1952) was used. The relative positions of the boundary, meniscus, and indices were measured on the photographic reproduction of the schlieren diagram by a travelling microscope, and measurements were reproducible to 0·005 cm. Sedimentation constants were obtained from the slope of the plot of $\Delta \log x$ against Δt, where x is the distance from the boundary to the centre of rotation and Δt the time between the exposures.

The density of 5M urea, 0·1M bisulphite, 0·05M phosphate (pH 8·3) relative to water was 1·09 that of 5M urea, 0·1M-NaCl, 0·05M phosphate (pH 6), 1·079, and that of 10M urea, 0·1M bisulphite, 0·05M phosphate (pH 9), 1·18. The viscosities of the solvents were measured at different temperatures relative to water at 20° and used together with the density measurements to obtain the sedimentation constants

corrected to the value they would have if the solvent were water at 20°. The partial specific volume was taken as 0·727 cm.³/g., this being the value found by Ward (1952) for wool keratin.

Light-scattering measurements were made in a light-scattering photometer (Brice, Halwer & Speiser, 1950) at 546 mμ. The keratin solutions were dialysed against the solvent and then centrifuged at 80000 *g* for 1 hr. They were then filtered through ultrafine sintered-glass filters (average pore diameter, 1·2 μ.) directly into the cell. Distilled water filtered directly through these filters had a turbidity of 2×10^{-5} cm.$^{-1}$, 5 M urea, 0·1 M bisulphite, 0·05 M phosphate (pH 8·5) had a turbidity of 9×10^{-5} cm.$^{-1}$. The concentrations of keratin were obtained after the measurements from the non-diffusible N contents. The values of HC/τ were calculated and plotted against concentration and molecular weights obtained from the value of HC/τ at zero concentration. H is the refraction constant, τ, the turbidity, is the logarithm of the fractional decrease in the transmitted intensity. C is the concentration of keratin in g./ml. (Precise definitions of H and τ are given by Oster (1948).

$$H = \frac{32\pi^3 n_0^2 (\Delta n/c)^2}{3N\lambda^4},$$

where n_0 is the refractive index of the solvent, $\Delta n/c$, the specific refractive increment, λ the wavelength of the incident light and N is Avagadro's number. The turbidity is defined by

$$I = I_0 e^{-x\tau},$$

I_0 is the intensity of the incident light, I the intensity of the transmitted light, and x the path length in the scattering system.)

RESULTS

Solubility and composition of soluble keratin

Fig. 1 illustrates the fractionation procedure.

With 10 M urea, 0·1 M bisulphite, 0·05 M phosphate as the solvent, extraction in the range pH 6–9·2 dissolved 80–85 % of the feather. 60 % was dissolved when 10 M urea, 0·5 M thioglycollate, 0·05 M phosphate (pH 6) was the solvent.

The extracts were yellow in colour. When the urea concentration at pH 6–8·5 was decreased to 5 M, the solutions developed a bluish opalescence due to the precipitation of a material which could be removed by high-speed centrifuging or passage through an ultrafine filter, and which represented a negligible fraction of the total weight of the keratin.

Decreasing the urea concentration in the extract made at pH 9 to 5 M, caused precipitation of part of the keratin as a fibrous clot. Rapid precipitation could be induced by overlaying the extract with an equal volume of 0·1 M bisulphite, 0·05 M phosphate

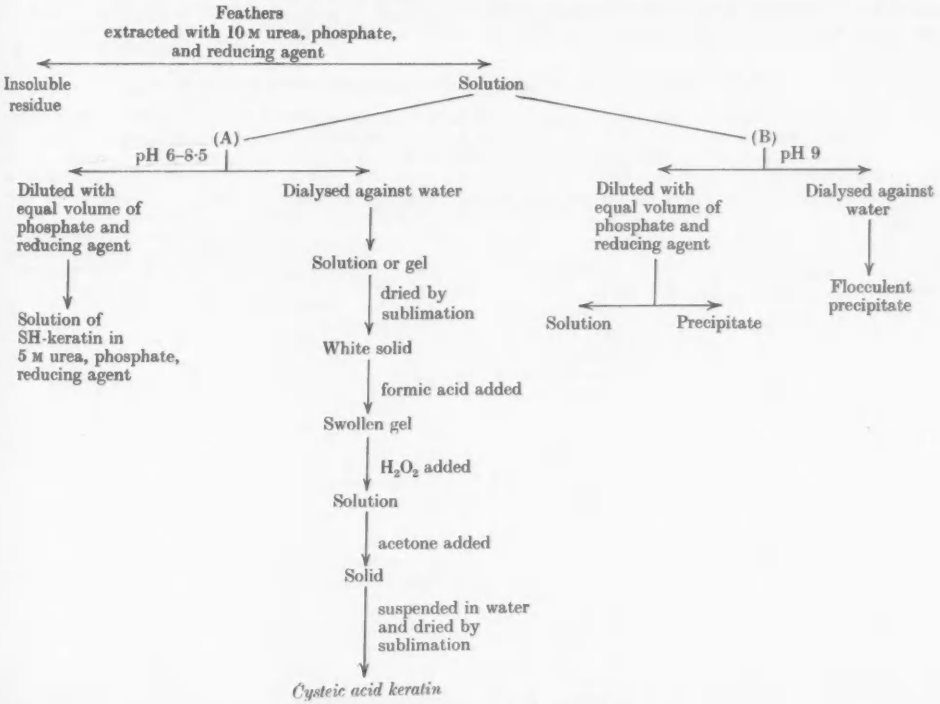


Fig. 1. Diagram of fractionation of feather keratin.

(pH 9), when an interfacial precipitate was produced, and then gently mixing the two layers. If, however, the solution of bisulphite and phosphate was added slowly and with stirring, a clear solution was obtained which developed an opalescence and then a precipitate during several hours. Precipitation in this way was always observed when the extracts were diluted immediately after separation from the insoluble residue. After standing at room temperature for several days, these extracts did not give a precipitate.

The extracts made in 10M urea, 0·1M bisulphite, 0·05M phosphate, (pH 6–8·5), when dialysed against water, gave a clear gel if the protein concentration was above about 3%, but an opalescent solution at lower protein concentration. The extract at pH 9 when dialysed in this way gave an opaque clot. The dialysed solutions and gels were frozen and dried by sublimation. The solid obtained from the gels, produced on dialysis of the extracts at pH 6–8·5, was soluble in 5M urea, 0·1M bisulphite, 0·05M phosphate (pH 6), but it was necessary to use 10M urea, 0·1M bisulphite, 0·05M phosphate, to induce solution at pH 8·5.

The cystine content of the solid recovered from the dialysed extracts was determined and the results are given in Table 1. There was loss of some of the cystine in the feather, but the loss was not dependent on the pH of the extraction. It is therefore unlikely that the precipitation observed on dilution of the extracts made at pH 9 is due to formation of thio-ether or other stable cross-links from the cystine residues. Middlebrook & Phillips (1942) found a small loss of combined cystine on treatment of wool with bisulphite at temperatures above 40°.

Table 2 lists the ratio of combined cysteine to combined cystine in soluble keratin under various conditions. The reaction with bisulphite is largely reversible; thus removal of the bisulphite from solutions of SH-keratin in 5M urea, 0·1M bisulphite,

Table 1. *Cystine contents of feather keratin and its derivatives*

In all cases the cysteine content was less than 0·3%. The percentages are calculated on the basis of the dry weight determined by heating at 105° but are not corrected for inorganic material remaining after the dialysis. The cystine content of the original feather is corrected for the moisture content and for the inorganic material present.

Material	pH of extraction	Cystine (%)
Feathers	—	7·6
Cysteic acid-keratin	—	Less than 0·2
SH-keratin from urea/bisulphite extraction after dialysis against water	6	6·9
	8·5	7·1
	9·2	6·9

Table 2. *Cysteine/cystine ratios of soluble keratin under various conditions*

Solutions in which the urea concentration was 5M were made from the extracts by dilution with 0·1M bisulphite, 0·05M phosphate, or with 0·5M thioglycollate, 0·05M phosphate. In all cases the solutions were dialysed against the solvent before the hydrolysis. The solid precipitated on dilution of the extract made in 10M urea, 0·1M bisulphite, 0·05M phosphate (pH 9) was washed in the solvent and suspended in that solvent before hydrolysis. The average number of —SH and —S—S— groups is calculated on the basis that each monomer has mol.wt. 10000 and potentially six —SH groups.

Material	Solvent	pH	Moles cysteine / Moles cystine	Average no. —SH groups in monomer	Average no. —S—S— groups in monomer
SH-keratin from urea/bisulphite extraction pH 6–8·5	5M urea, 0·1M bisulphite, 0·05M phosphate	6	10–14	5·2	0·4
		8·5	1·0–1·4	2·2	1·9
	5M urea, 0·1M bisulphite, 0·05M phosphate, 0·2M ethanol	8·5	1·4	2·4	1·8
	10M urea, 0·1M bisulphite, 0·05M phosphate	6	>25	6	0
		8·5	4	4	1
		9·2	3·4	3·8	1·1
	5M urea, 0·1M-NaCl, 0·05M phosphate	6	0·08	0·2	2·9
SH-keratin remaining in solution after dilution of the extract in 10M urea (pH 9)	5M urea, 0·1M bisulphite, 0·05M phosphate	9·2	2·0	3	1·5
Solid precipitated on dilution of the extract in 10M urea (pH 9)	5M urea, 0·1M bisulphite, 0·05M phosphate	9·2	0·23	0·6	2·7
SH-keratin from urea/thioglycollate extraction (pH 6)	5M urea, 0·1M-NaCl, 0·05M phosphate	5·8	0·13	0·4	2·8

0·05M phosphate (pH 6) by dialysis against 5M urea, 0·1M-NaCl, 0·05M phosphate (pH 6) resulted in loss of many of the free —SH groups. In an attempt to prepare a solution of SH-keratin with a large number of free —SH groups in the absence of reducing agent, a solution of SH-keratin was prepared in 5M urea, 0·5M thioglycollate, 0·05M phosphate (pH 5·5) and dialysed against 5M urea, 0·1M-NaCl, 0·05M phosphate, 0·1% (pH 5·5), but this too resulted in a product with many —S—S— bonds.

The cysteine/cystine ratio of SH-keratin in urea and bisulphite solution is dependent on the pH and the urea concentration. While the effect of both could be to change the reactivity of the —S—S— groups, it is probable that the effect of the pH, in part at least, is to change the concentration of the HSO_3^- ion which, according to Lugg (1932), reacts with disulphides by the reaction

$$R—S—S—R + HSO_3^- \rightleftarrows R—S—H + R—S—SO_3^-.$$

Solutions of bisulphite titrate in the range pH 4–9. The reaction with alkali is

$$HSO_3^- + OH^- \rightleftarrows SO_3^{2-} + H_2O$$

and there is very little HSO_3^- at pH 9. Elsworth & Phillips (1932) found that the number of free —SH groups in wool, suspended in buffered solutions of bisulphite, was similarly dependent on the pH and concluded that it was due to a change in the concentration of the HSO_3^- ion.

Cysteic acid-keratin was soluble in 5M urea, 0·1M-NaCl, 0·05M phosphate (pH 6–8·5) and in 0·05M-Na_2CO_3 or in 0·05M-Na_2CO_3, 0·2M-NaCl. A 2% (w/v) solution of cysteic acid-keratin in 0·05M-Na_2CO_3 migrated with a single boundary on electrophoresis. This is consistent with there being an equal distribution of cysteic acid side chains, and hence the cystine, among the keratin monomers.

Osmotic pressure

Table 3 gives the values of P/C at zero concentration, the number-average molecular weight, and also the ratio of P/C at zero and 1% concentrations, for soluble keratin under various conditions. Figs. 2–4 record the experimental data for some of these conditions.

Table 3. *Results of osmotic pressure measurements*

When the urea concentration was 5M, the extract was diluted with 0·1M bisulphite, 0·05M phosphate of the appropriate pH and this solution was then dialysed against the solvent used in the osmometer. P = pressure in mm. Hg; C = concentration of keratin in g./100 ml. solution.

Solute	Solvent	pH	P/C ($C=0$)	Mol.wt.	$\frac{P/C\ (C=0)}{P/C\ (C=1)}$
Cysteic acid-keratin	5M urea, 0·1M-NaCl, 0·05M phosphate	6	18·2	10 200	1
	5M urea, 0·1M bisulphite, 0·05M phosphate	8·5	18·2	10 200	1
SH-keratin from extraction in urea/bisulphite	5M urea, 0·1M bisulphite, 0·05M phosphate	6	18·2	10 200	1·08
		8·5	19·7	9 500	1·45
	5M urea, 0·1M bisulphite, 0·115M phosphate	8·5	19·8	9 500	1·54
	5M urea, 0·1M bisulphite, 0·05M phosphate, 0·2M ethanol	8·5	19·8	9 500	1·58
	5M urea, 0·1M bisulphite, 0·05M phosphate, 0·2M mercaptoethanol	8·5	19·2	9 700	1·09
	5M urea, 0·1M bisulphite, 0·05M phosphate, 0·2M thioglycollate	8·5	18·5	10 100	1·08
	10M urea, 0·1M bisulphite, 0·05M phosphate	9·2	18·5	10 100	1·25
SH-keratin from urea/thioglycollate extraction	5M urea, 0·1M-NaCl, 0·05M phosphate	5·8	19·0	9 800	1·4

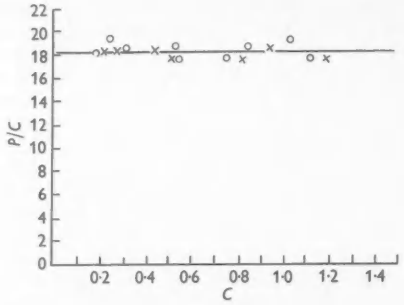


Fig. 2. Osmotic pressure of cysteic acid-keratin. ○, 5M urea, 0·1M-NaCl, 0·05M phosphate (pH 6); ×, 5M urea, 0·1M bisulphite, 0·05M phosphate (pH 8·5). P = pressure in mm. Hg; C = concn. of keratin in g./100 ml. solution.

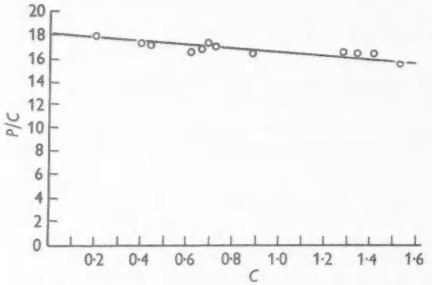


Fig. 3. Osmotic pressure of SH-keratin in 5M urea, 0·1M bisulphite, 0·05M phosphate (pH 6). P = pressure in mm. Hg; C = concn. of keratin in g./100 ml. solution.

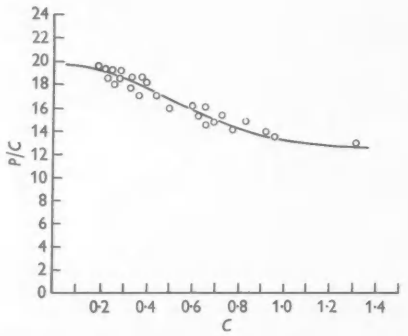


Fig. 4. Osmotic pressure of SH-keratin in 5M urea, 0·1M bisulphite, 0·05M phosphate (pH 8·5). P = pressure in mm. Hg; C = concn. of keratin in g./100 ml. solution.

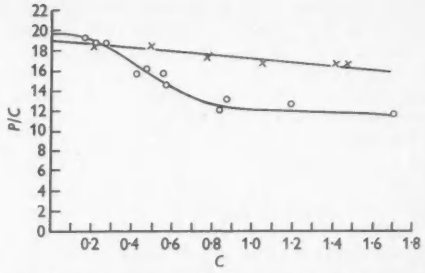


Fig. 5. Osmotic pressure of SH-keratin. ○—○, 5M urea, 0·1M bisulphite, 0·05M phosphate, 0·2M ethanol (pH 8·5). ×—×, 5M urea, 0·1M bisulphite, 0·05M phosphate, 0·2M β-mercaptoethanol (pH 8·5). P = pressure in mm. Hg; C = concn. of keratin in g./100 ml. solution.

The graphs representing the dependence of P/C on concentration all extrapolate, with different slopes, to the same value of $P/C = 19 \pm 1$ at zero concentration. Thus the number-average molecular weight of soluble keratin is constant in the urea concentration range 5–10M, in the range of ionic strength 0·17–0·64 and for preparations which have 0–2 moles of cystine/10000 g.

Cysteic acid-keratin gives values of P/C which are independent of concentration in the range studied. SH-keratin deviates from ideality, indicating that association occurs with increasing concentration. The extent of the association is represented by the ratio of P/C at zero to that at 1% concentration and comparison with the cystine content of the material suggests that the association only occurs when —S—S— bonds are present. However, cysteic acid-keratin, SH-keratin at pH 6, and SH-keratin at pH 8·5, are not strictly comparable, as they will have different net charges, and the ionic strength of the media in which the measurements were made was not constant. It was possible that the association was due to a charge effect. However, the addition of β-mercaptoethanol decreases the association of SH-keratin at pH 8·5, while the addition of ethanol does not (Fig. 5), and so the association probably requires the presence of —S—S— bonds.

SH-keratin associates with increasing concentration in 5M urea, 0·1M bisulphite, 0·115M phosphate (pH 8·5), but in this solution, which has the highest ionic strength which has been used, precipitation of part of the keratin occurred when the protein concentration was above 2%.

Viscosity

Fig. 6 shows the relation between the specific viscosity, $(\eta_{rel.} - 1)/C$, and concentration for cysteic acid-keratin in 5M urea, 0·1M-NaCl, 0·05M phosphate (pH 6) and for SH-keratin in 5M urea, 0·1M bisulphite, 0·05M phosphate (pH 8·5). Extrapolation

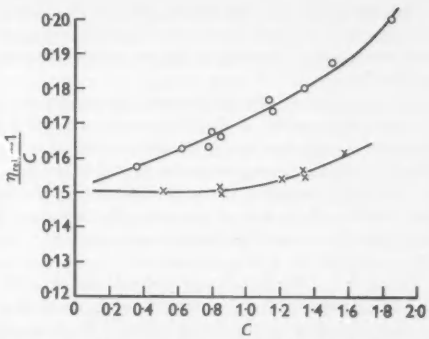


Fig. 6. Viscosity results. ○—○, SH-keratin in 5 M urea, 0·1 M bisulphite, 0·05 M phosphate (pH 8·5). ×—×, Cysteic acid-keratin in 5 M urea, 0·1 M-NaCl, 0·05 M phosphate (pH 6).

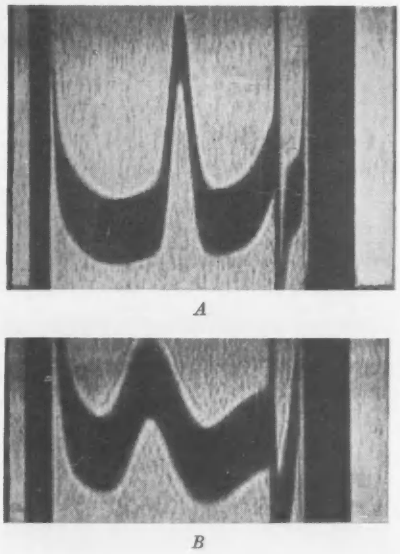
of these graphs to zero concentration gives the same value for the intrinsic viscosity (specific viscosity at zero concentration) for both preparations. Taking the partial specific volume as 0·727, the viscosity increment is 20·6. The viscosity increment is $(\eta_{rel.}-1)/\phi$; $\phi=0$, where ϕ is the volume fraction of the keratin. If the keratin particle is a prolate ellipsoid and unsolvated, then the corresponding value for the axial ratio is 13·2 (Simha, 1940). If the shape is spherical, the viscosity increment should be 2·5 (Einstein, 1906, 1911) and it would be necessary to assume that the keratin is solvated to a volume 8 times that of the partial specific volume. Pasynskiĭ & Chernyak (1950) have measured the absorption of water and urea by wool keratin, using the technique of equilibrium dialysis, and give the values 0·15 and 0·08 g./g. keratin, respectively. It is therefore probable that the viscosity of keratin is determined mainly by an asymmetric shape.

At concentrations below 1 % the specific viscosity of cysteic acid-keratin is independent of concentration but the specific viscosity of SH-keratin at pH 8·5 increases with concentration. It is unlikely that the hydration would vary with protein concentration and so the increase in viscosity is taken as evidence for formation of increasingly asymmetric molecules by association.

A further feature of interest in the dependence of the specific viscosity on concentration is that the curve for SH-keratin at pH 8·5 does not flatten at concentrations above 1 %, at which level P/C becomes independent of concentration.

Sedimentation

Fig. 7 shows the schlieren diagram for SH-keratin at pH 8·3 in 5 M urea, 0·1 M bisulphite, 0·05 M phosphate, sedimenting in the centrifugal field (250 000 *g*). Fig. 8 gives the diagram for



A

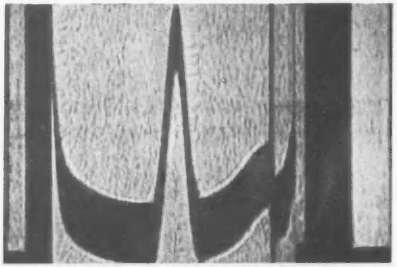
B

Fig. 7. Schlieren diagrams of SH-keratin sedimenting in the centrifugal field. The solvent was 5 M urea, 0·1 M bisulphite, 0·05 M phosphate (pH 8·3), and the keratin concentration 0·8 % (w/v). *A*, 20 min. after forming the boundary. *B*, 131 min. after forming the boundary. (Sedimentation from right to left.)

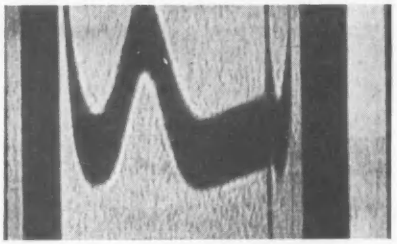
cysteic acid-keratin in 5 M urea, 0·1 M-NaCl, 0·05 M phosphate (pH 6). That for SH-keratin in 10 M urea, 0·1 M bisulphite, 0·05 M phosphate (pH 9) was indistinguishable from these.

Cysteic acid-keratin and SH-keratin sediment with a single symmetrical boundary. The slow rate of sedimentation does not permit this to be taken as evidence for a single sedimenting species, but it is evidence that there is not present in solution a mixture of monomer with stable polymers, unless the polymers have a shape which compensates for their increased weight and so have a sedimentation rate identical with the monomer. Alternatively, since it is known from the osmotic pressure measurements that SH-keratin is associated under these conditions, the appearance of the sedimentation boundary could arise if the protein were sedimenting as an equilibrium mixture of monomer and polymer in which the rate of association and disassociation were fast compared with the rate of sedimentation.

Fig. 9 gives the relation between the sedimentation constant and concentration for cysteic acid-keratin, and Table 4 gives the values of the sedimentation constant at different concentrations of SH-keratin in 5 M urea (pH 8·3) and for SH-keratin



A



B

Fig. 8. Schlieren diagrams of cysteic acid-keratin sedimenting in the centrifugal field. The solvent was 5 M urea, 0·1 M-NaCl, 0·05 M phosphate (pH 6), and the concentration of the keratin 1·8% (w/v). *A*, 30 min. after forming the boundary; *B*, 176 min. after forming the boundary. (Sedimentation from right to left.)

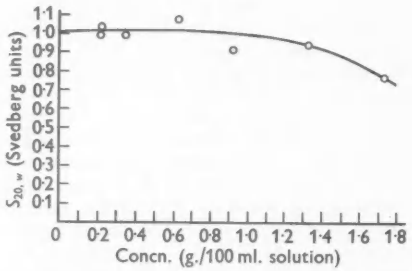


Fig. 9. Sedimentation constants of cysteic acid-keratin in 5 M urea, 0·1 M-NaCl, 0·05 M phosphate (pH 6).

in 10 M urea (pH 9). Within the limits of the experimental error, the variation of sedimentation constant with concentration is the same for all three preparations.

The identity of the sedimentation constants of cysteic acid-keratin and SH-keratin at pH 8·3, considered together with the association that the osmotic pressure measurements reveal in solutions of the latter, requires that the association should occur with production of increasingly asymmetric particles. The viscosity measurements give qualitative support for this explanation.

From the sedimentation constant and the frictional ratio the molecular weight can be calculated by the equation given by Svedberg & Pedersen (1940). The frictional ratio of keratin, obtained from the axial ratio by Perrin's equation (Perrin, 1936) is 1·7 and the corresponding value of the molecular weight of cysteic acid-keratin is 10100.

If, however, the ratio of the molecular weights of SH-keratin and cysteic acid-keratin, at 1% concentration, is calculated from the appropriate frictional ratios the value of 1·15 is obtained. The ratio of the number-average molecular weights at this concentration is 1·5 and this discrepancy cannot be due to experimental error alone.

Light-scattering

Fig. 10 gives the relationship between HC/τ and concentration of SH-keratin in 5 M urea, 0·1 M bisulphite, 0·05 M phosphate at pH 6 and

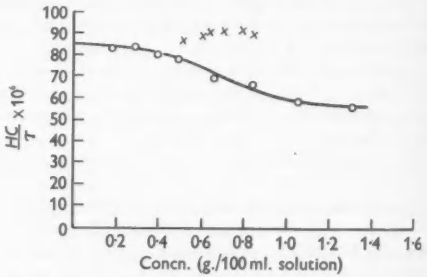


Fig. 10. Light scattering by SH-keratin. × × ×, In 5 M urea, 0·1 M bisulphite, 0·05 M phosphate (pH 6). O—O, In 5 M urea, 0·1 M bisulphite, 0·05 M phosphate (pH 8·5).

Table 4. *Sedimentation constants for* SH-*keratin*

Solute	Solvent	pH	Concn. (%, w/v)	$S_{20,w}$
SH-keratin extracted at pH 8·3	5 M urea, 0·1 M bisulphite, 0·05 M phosphate	8·3	1·5	0·97
			1·25	0·97
			0·7	1·00
			0·3	0·93
SH-keratin extracted at pH 9	10 M urea, 0·1 M bisulphite, 0·05 M phosphate	9·1	3·0	0·63
			1·2	0·94
			0·67	0·93

pH 8·5. The weight-average molecular weight is 11 000 ± 1000. This is sufficiently close to the number-average molecular weight (10 000) to warrant the conclusion that there is not a large distribution of particle weights. Deviations of the molecular weight obtained in this way from the true value could occur due to preferential absorption of urea or water (Ewart, Roe, Debye & McCartney, 1946, Doty & Edsall, 1951), but as Pasynskiĭ & Chernyak (1950) found little absorption of either on wool keratin, they probably do not occur with feather keratin.

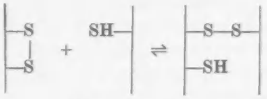
The variation of the turbidity with concentration of SH-keratin at pH 8·5 is taken as confirmation of the association detected by the osmotic pressure measurements.

DISCUSSION

As it is known that keratin in the solid state is aggregated by —S—S— bonds, hydrogen bonds and salt linkages, it is necessary, in order to identify the keratin monomer, to specify the extent to which these forces are present between the keratin particles in the solutions in which the measurements of molecular size were made. It is probable that solutions of cysteic acid-keratin and infinitely dilute solutions of SH-keratin in 5 M urea, 0·1 M bisulphite, 0·05 M phosphate (pH 6–8·5), contain no intermolecular bonding of this sort, for cysteic acid-keratin which has no —S—S— bonds and SH-keratin which does have them have the same value for the number-average molecular weight, and increase of the urea concentration from 5 M (pH 8·5), to 10 M (pH 9) or variation of the ionic strength from 0·17 (pH 6) to 0·64 (pH 8·5) does not change that value.

With regard to the effect of the ionic strength, in solutions with a pH different from the isoelectric point there will be a repulsive force between the keratin particles and the increase of ionic strength would reduce that force. Alternatively, if there were a considerable distance between electrically positive and negative centres, attraction between the oppositely charged parts of adjacent molecules would be possible and the effect of high ionic strength would be to decrease the interaction. The constancy of the molecular weight under the conditions listed in Table 3 is taken as evidence for the absence of aggregation due to these effects.

It must therefore be concluded that the —S—S— bonds which are present in SH-keratin are, when the solutions are very dilute, intramolecular. The association which occurs with increasing concentration can be due to formation of intermolecular —S—S— bonds at the expense of intramolecular bonds through the intermediary action of the SH groups, of which only a catalytic amount would be necessary.



Dissociation would be favoured by the mass-action effect of dilution. Association may be favoured by the increased opportunity which polymer formation affords for hydrogen bonding between the polypeptide chains. Indeed, it is probable that some secondary bonding is a necessary condition for the association to occur since it produces molecules of increased asymmetry and there will be a decrease in the entropy of the system. This explanation of the association is analogous to that put forward by Huggins, Tapley & Jensen (1951), to account for the formation of gels by concentrated solutions of serum albumin in urea and thioglycollate.

We have little information on the homogeneity of the polypeptides in soluble feather keratin except that the ultracentrifuging and a single electrophoresis experiment are consistent with there being a narrow distribution of particle weights and charge. However, it has been shown by end-group analysis that wool keratin contains several polypeptides of similar weights (Middlebrook, 1951), and there is no evidence to show that this is not also true of feather keratin. The precipitation of some SH-keratin at high ionic strength or on diminution of the urea concentration at pH 9, cannot be taken as evidence for chemical heterogeneity, as in the former case the precipitation is dependent on the protein concentration and in the latter on the age of the solutions.

The solubility of SH-keratin may be determined by the configuration which the polypeptide chains assume (cf. Blackburn, 1951). Pauling & Corey (1953) have described possible structures for keratins, and the different degrees of hydrogen bonding in these may determine their relative stability in the solid state. Alexander & Earland (1950) found a correlation between the solubility of a product obtained by oxidation of wool and its ability to give an α or β X-ray diagram.

The graph relating the specific viscosity to concentration of SH-keratin at pH 8·5 does not flatten at concentrations above 1 % in the way found with the P/C against C relationship. Cysteic acid-keratin is ideal over this concentration range, but this need not be true of the polymers formed by association of SH-keratin. Indeed, because of their increased asymmetry, they would be expected to deviate from ideal behaviour at lower concentrations than the monomer, and the increasing slope of the specific viscosity/concentration relationship indicates that this is so.

For this reason it is not possible from the osmotic pressure measurements to set an upper limit to the association. Molecular weights can only be obtained from osmotic pressure measurements at finite concentrations when the solutions are ideal, that is the entropy of mixing is given by $\Delta S = -\boldsymbol{R} \ln N$, where N is the mole fraction of the solvent. For asymmetric molecules this relationship does not hold and above a certain concentration P/C becomes dependent on concentration (Flory, 1945; Huggins, 1943). For feather keratin, deviations from ideality from this cause would operate in the opposite way from deviations due to the association.

Similar considerations apply to the variation of the turbidity with concentration. The turbidity of a solution of finite concentration is given by

$$\frac{HC}{\tau} = \frac{1}{M} + \frac{2BC}{\boldsymbol{RT}},$$

where H, C and τ have the definition already given, M is the molecular weight, $\boldsymbol{R}$ the gas constant, T the absolute temperature, and B is a constant depending on the shape of the solute molecules, their interaction with the solvent, and with each other. At finite concentrations of asymmetric molecules, B operates to produce an increase of HC/τ with concentration; this is in the opposite sense to the effect of the association.

However, although the extent to which association proceeds cannot be specified from the experiments described here, the formation of gels on dialysis of solutions of SH-keratin indicates that association can proceed beyond the dimer level. If hydrogen bonding with urea and electrostatic repulsion between the keratin monomers tend to decrease the extent of association, then a lower urea concentration and a higher ionic strength may permit the formation of larger, but soluble, polymers than those detected in the solutions used in this work. Physical measurements on such solutions might give additional support to the hypothesis put forward here, that solutions of keratin with both —SH and —S—S— groups contain an equilibrium mixture of monomer and polymer in which the polymerization has produced increasingly asymmetric particles. With such solutions it might be possible to obtain better agreement between the calculated and measured sedimentation constants of keratin at finite concentrations.

SUMMARY

1. The preparation of solutions of feather keratin by dispersion in urea and bisulphite and by oxidation with performic acid is described.

2. The cysteine/cystine ratio of keratin dissolved in solutions of urea and bisulphite depends on the urea concentration and on the pH.

3. The physical properties of keratin in solution have been measured and correlated with the cystine and cysteine contents.

4. Measurements of osmotic pressure, turbidity, sedimentation rate and viscosity indicate that in infinitely dilute solution keratin has a molecular weight of 10000 and an axial ratio of 13.

5. Preparations which contain both free —SH groups and —S—S— bonds associate with increasing concentration. It is concluded that the association involves the formation of intermolecular —S—S— bonds at the expense of intramolecular bonds.

6. Association produces a particle which is more asymmetric than the monomer.

7. Keratin sediments with a single boundary in the ultracentrifuge.

8. The variation of the viscosity, osmotic pressure, sedimentation rate and turbidity with concentration of dissolved keratin is discussed.

I wish to thank Prof. K. J. Mysels for his advice and interest in this work, which was carried out during the tenure of the Rubberset Fellowship of the University of Southern California and a Fulbright Travel Grant.

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The Effect of Anionic Detergents and some Related Compounds on Enzymes

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Synthetic detergents of all types are now widely used domestically, and in many branches of medicine and industry. The biological effects of these detergents are only partially understood, and it was considered that a systematic examination of the effect of some of these detergents on enzymes would be of value. Most commercial detergents are complex mixtures and often of secret formula, but some classifications have been presented in the literature (e.g. Van Antwerpen, 1943). The present work deals only with those detergents usually referred to as the 'anionic detergents'.

In earlier investigations from this laboratory it was found that the trypanocidal drug Suramin has a strong inhibitory action on a few enzymes at pH 7·5 and a complete or almost complete inhibiting effect on many enzymes on the acid side of their isoelectric points (Wills & Wormall, 1950*a*, *b*; Wills, 1952*a*). As an extension of this work, a study has now been made of the effect of various detergents on enzymes, since preliminary experiments indicated that some detergents have an effect on enzymes somewhat similar to that of Suramin. In the investigations described here a few experiments have been made with commercial detergents, but in most cases sodium dodecyl sulphate (SDS), which is obtainable in a fairly pure state, has been used as a typical anionic detergent.

Several studies have been made of the combination of SDS with proteins (Putnam & Neurath, 1944), but few observations have been made on the effect of SDS or related compounds on enzymes. Marron & Moreland (1939) reported that surface-active substances (including a detergent) had little effect on several enzymes tested. SDS has been observed to precipitate crystalline pepsin at acid pH (Putnam & Neurath, 1944) and inactivate it at pH 4·0 (Cooper & Bull, 1944) but only slight combination of this enzyme and detergent occurs at pH 6·5 (Lundgren, 1945). More recently, Rabinovitz & Boyer (1950) have shown that SDS inhibits the succinic oxidase system.

In the present work the effect of SDS on enzymes has been compared with the effect of several aromatic sulphonic acids and with that of some commercial detergents which are sulphonated compounds, e.g. Teepol. A preliminary report on some of these investigations has been presented (Wills, 1952*b*, 1953).

EXPERIMENTAL

Enzyme preparations and activity measurements

The methods of preparation and estimation of activity for most of the enzymes used have been previously described (Wills & Wormall, 1950*b*). Other sources and methods are described below. Where SDS or another substance was being tested for inhibitory power, a suitable concentration of this test substance was added to the enzyme mixture and the amount of water reduced accordingly.

Lipase. This was used as an aqueous extract of hog pancreas powder. This powder was prepared by extracting a pancreas with acetone and ether, and drying and pulverizing the solid in a mechanical mortar. A 5 g. sample of the powder was extracted with 50 ml. water in a mechanical shaker, and the supernatant centrifuged.

D-*Amino acid oxidase.* An aqueous extract of dried acetone-powder of pig kidney prepared as described by Krebs (1935) was used.

Lipase estimations. These were made by three methods.

(i) *Continuous-titration method.* Water (3·8 ml.), triacetin (0·2 ml.) and bromothymol blue (4 drops of a 0·04% solution) were mixed in a boiling tube at 18° and the pH was adjusted to 6·8 with 0·01 N-NaOH. Enzyme solution (1 ml.) was added and the pH kept at 6·8 by addition of 0·01 N-NaOH. Titration readings were taken at intervals of 1 or 2 min. for about 10 min.

(ii) *Manometric method.* The enzyme action was studied at 37° by following acid production as recorded by liberation of CO_2 from $NaHCO_3$. The manometers were gassed with either 95% N_2—5% CO_2 or with 100% CO_2, much more concentrated $NaHCO_3$ soln. being necessary in the latter case. By adjusting the concentration of $NaHCO_3$ the pH of the mixture was varied, the precise concentration of $NaHCO_3$ necessary to give the required pH being calculated by means of the Henderson-Haselbalch equation (cf. Umbreit, Burris & Stauffer, 1949). 100% CO_2 proved more suitable for acid pH's.

For pH 7·4, $NaHCO_3$ solution (1 ml. of 0·06 M for 5% CO_2 or 0·85 ml. of 1 M for 100% CO_2), enzyme solution (1 ml.) and water (1·0 ml.) were placed in the main compartment of the Warburg flask and either neutralized triacetin (0·3 ml.) or neutralized triolein (0·4 ml.) in the side bulb.

(iii) *Willstätter's method.* This is described by Sumner & Somers (1947) and was used at 37°.

Trypsin estimations

(i) *Manometric method.* 0·06 M-$NaHCO_3$ (1 ml.), trypsin solution (1 ml.), and water (1 ml.) were placed in the main compartment of a Warburg flask and 5% (w/v) casein (0·3 ml.) in the side bulb. The gas phase was 95% N_2—5% CO_2 and rate of CO_2 liberation was measured at 37°.

(ii) *Titration method.* Digestion was followed by the formol-titration method: 5% casein (15 ml.) or serum (15 ml.), water (20 ml.) and trypsin solution (10 ml.) were mixed in a conical flask which was placed in a water bath at 37°. Samples (10 ml.) were withdrawn at 0, 30, 60 and 90 min. and blown into 20% (v/v) formalin (10 ml.) at pH 8·2. Phenolphthalein solution (1 drop/ml.) was added and the samples were titrated to pH 8·2 with 0·05 N-NaOH. The amount of NaOH required gave a measure of the progress of the digestion.

Invertase estimations. These were (i) as previously described (Wills & Wormall, 1950*b*), and (ii) polarimetrically. In the latter method, N acetate buffer, pH 4·0 (2·0 ml.), 20% (w/v) sucrose (20 ml.), and water (23 ml.) were mixed in a conical flask which was placed in a water bath at 37°. The reaction was started by the addition of invertase solution (5 ml.). Samples (10 ml.) were withdrawn after 0, 10, 20, 30 and 40 min. and quickly introduced into 2 N-NaOH (5 ml.). The rotation of each sample was then measured in the polarimeter.

Materials. Commercial detergents were obtained from the following sources: Teepol (the sodium salt of higher alkyl sulphates) from Shell Chemicals Ltd., Liquoide Roche (sodium polyanetholsulphonate) from Roche Products Ltd., and Stergene (an alkylarylpolyethoxyethanol) form Domestos Ltd.

Antrypol amine (*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-aminonaphthalene-4:6:8-trisulphonic acid) was a gift from I.C.I. Ltd.

RESULTS

Inhibition of urease by sulphonic acids

The effect of various structurally related sulphonic acids on urease was studied at pH 5·0. The compounds were tested in concentrations of 0·001 M and 0·00033 M and all appeared to fall fairly sharply into one of two groups. Compounds termed type *A* and listed in Table 1 were found to be very weak inhibitors of urease even at a concentration of 0·001 M. A consideration of the structures of these compounds shows that although these vary greatly there is one common characteristic, namely that all these compounds possess only one sulphonic acid grouping or two or more closely packed sulphonic acid groupings (as for example in the naphthalene ring). When inhibition was produced by type *A* compounds it was reversible with change of pH, so that adjusting the pH to 5·7 instantly abolished the inhibition.

The remaining compounds tested are termed type *B* and are listed in Table 2. These sulphonic acids, in contrast to type *A* compounds, are powerful inhibitors of urease in a concentration of 0·00033 M and even in much weaker solutions. Type *B* compounds also vary considerably in structure, amongst themselves, but they all appear to have a common characteristic in possessing two or more sulphonic acid groups which are 'spaced' or well separated by other groups as illustrated in the formulae given in Table 2. Whether there is one or more sulphonic acid groups at each end of the molecule has no important effect on the inhibitory power, nor has the nature of the intervening part of the molecule which separates the sulphonic acid groups. Type *B* compounds give inhibition which is irreversible with change of pH, and give inhibition/pH curves of the type given by Suramin. Suramin, which has already been extensively investigated (Wills & Wormall, 1950*b*), belongs to this group of type *B* compounds, but it will be noticed that the half molecule of Suramin (Antrypol amine) and the terminal residue (1-naphthylamine-4:6:8-trisulphonic acid) of Suramin are both very weak inhibitors of urease and thus belong to type *A*.

The effect of SDS on urease

Interpretation of the results of examining the effect of SDS on urease is greatly facilitated by considering the behaviour of the SDS molecule in solution.

Micelle formation of SDS. Physico-chemical investigations of SDS solutions, by measurement of electrical conductivity, osmotic pressure and by other methods, have shown that the molecule of SDS may exist in solution either as a free ion or as a micelle. In dilute solutions, SDS exists mainly as the free ion, but in concentrated solutions the micelle predominates. This is thought to be approximately spherical and consist of several associating molecules of SDS with a negatively charged surface layer (cf. Hartley, 1936; Ralston, 1946). The transition between the two forms is fairly sharp and occurs at a critical concentration which depends on the salt concentration. For the buffer concentration used in most enzyme experiments, it is approximately 0·001 M. Transition in either direction is not 100%, and there is evidence that some micelles exist even in dilute solutions.

Table 1. *Effect of aromatic sulphonic acids and SDS on urease at* pH 5·0

Type *A* compounds (all except SDS used in a concentration of 10^{-3} M). For concentration of SDS—see text, p. 110.

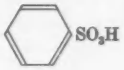
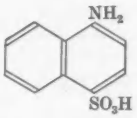
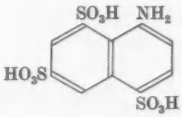
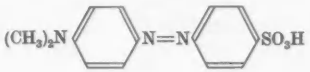
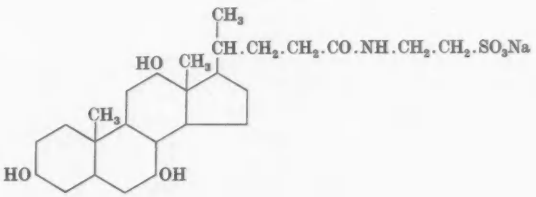
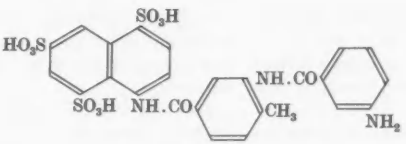
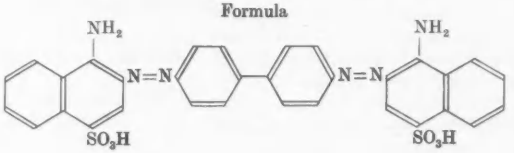
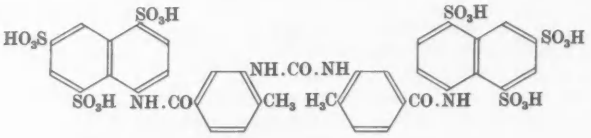
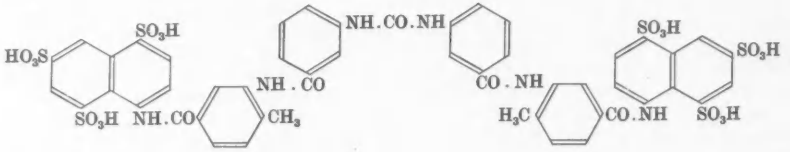
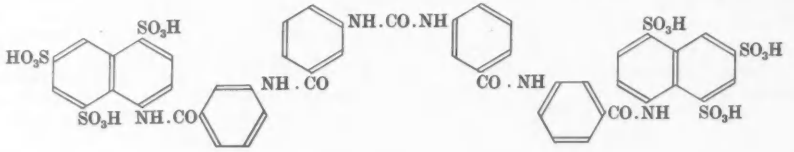
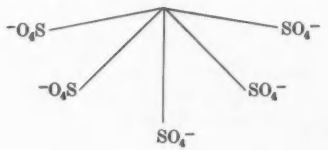
Compound	Formula	Inhibition (%)
Benzenesulphonic acid		0
p-Toluenesulphonic acid		0
Sulphanilic acid		0
Naphthionin		0
Suramin terminal group (1-naphthylamine-4:6:8-trisulphonic acid)		25
Methyl orange		0
Sodium taurocholate		0
Antrypol amine		0
SDS ion	$CH_3.(CH_2)_{11}.SO_4^-Na^+$	0

Table 2. *Effect of aromatic sulphonic acids and SDS on urease at* pH 5·0

Type *B* compounds (all except SDS used in a concentration of 10^{-3} M). For concentration of SDS—see text.

Compound	Formula	Inhibition (%)
Congo red		90
		90
Suramin		90
'Demethylated' Suramin		90
SDS micelle		90

Results of experiments which are presented later in this paper show that, in their behaviour towards urease, the SDS ion closely resembles type *A* compounds and the SDS micelle resembles type *B* compounds.

The effect of concentration. At pH 5·0 urease is completely inhibited by 0·001 M-SDS, but as the concentration of SDS is reduced inhibition falls off rapidly until it is almost zero with 0·00033 M-SDS. Comparing these results with those obtained for Suramin in equivalent concentration (Fig. 1) it will be observed that the rate of fall of inhibition with decreasing concentration is much more rapid with SDS than it is in the case of Suramin.

The effect of pH. SDS (0·001 M) causes 100% inhibition of urease at pH 5·0, but the inhibition is markedly dependent on pH as it is in the case of Suramin (Wills & Wormall, 1950*b*) and falls to zero at pH 5·4. An inhibition/pH curve is obtained (Fig. 2) practically identical with that obtained using Suramin.

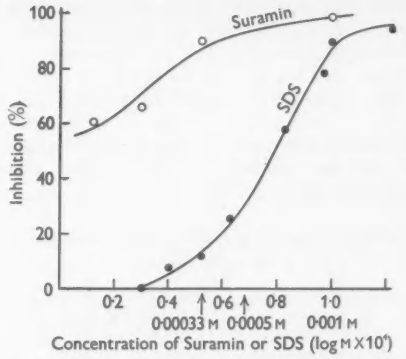


Fig. 1. Comparison of the effect of varying concentrations of SDS and Suramin on urease at pH 5·0.

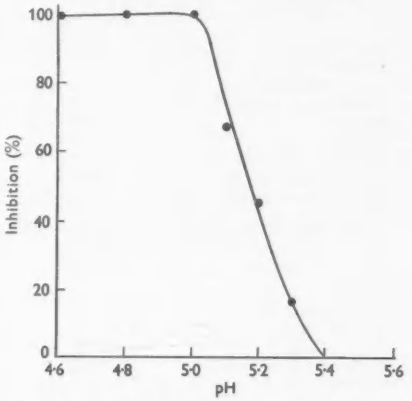


Fig. 2. Effect of pH on inhibition of urease by 0·001 M-SDS.

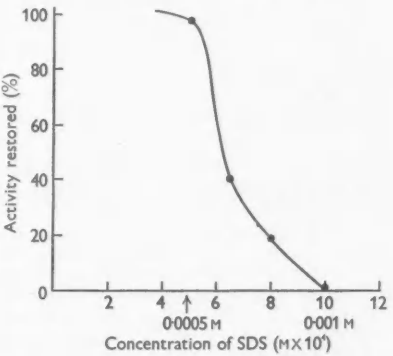


Fig. 3. Reversibility of SDS inhibition of urease with change of pH from 5·0 to 5·7. Urease and SDS were left in contact for 20 min. at pH 5·0, and the estimation was carried out at pH 5·7.

Reversibility of inhibition. Inhibition of urease by Suramin at pH 5·0 has been shown to be irreversible with change of pH, in that the enzymic activity of urease treated with Suramin at pH 5·0 cannot be restored by adjusting the pH to 5·7, a pH at which urease is not normally inhibited by Suramin (Wills & Wormall, 1950*b*). Other compounds of type *B* were found to behave like Suramin and produce inhibition which was not reversed by change of pH. Inhibition produced by the 'terminal residue' of Suramin (type *A* compound) was found, however, to be completely reversible when the pH was adjusted from pH 5·0 to 5·7, inhibition by 0·001 M being 37 % at pH 5·0 and zero at pH 5·7, whereas inhibition by 0·001 M Suramin is 100 % at both pH's.

In this respect SDS showed a transition between type *A* and type *B* properties. The inhibition was quite irreversible when 0·001 M-SDS was used but it was completely reversible at 0·0005 M (Fig. 3). Since in a concentration of 0·001 M, SDS will be almost completely in the micellar form and almost completely dissociated at 0·0005 M, these results indicate that inhibition produced by the micelle is irreversible (type *B*), but that produced by the free ion is reversible (type *A*).

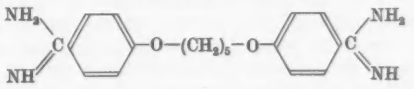
The effect of other surface-active agents on urease

In addition to SDS the effect of some other anionic surface-active agents on urease was examined. These included Teepol, Liquoide Roche, and sodium taurocholate.

Teepol and Liquoide Roche were found to be powerful inhibitors of urease at pH 4·8; thus 0·1 % (v/v) Teepol (commercial preparation) gave 100 % inhibition, and 0·1 % (w/v) Liquoide Roche gave 95 % inhibition. In both cases inhibition fell off sharply with increasing alkalinity, and pH/inhibition curves similar to that given with SDS were obtained.

Sodium taurocholate was found to be a weak inhibitor; 0·3 % (0·061 M) produced 23 % inhibition at pH 5·0 and it gave no sharp pH/inhibition curve.

The effect of pentamidine



Pentamidine, 4:4′-diamidinodiphenoxypentane, which like Suramin is a trypanocidal drug, was observed by Guimaraes & Lourie (1951) to give a marked precipitate when mixed with Suramin, as a consequence of which the trypanocidal effect of both drugs was abolished. These experiments suggested that it might prove possible to protect enzymes against inhibition by Suramin with pentamidine.

Table 3. *Effect of* 0·001 M *pentamidine on urease treated with various concentrations of Suramin or SDS at* pH 5·0, *for varying lengths of time*

Material	Concn. (M)	Time of contact with enzyme before addition of pentamidine (min.)	Inhibition (%)
Suramin	0·00033	No pentamidine added	72
		0	0
		0·25	0
		5	30
		10	42
		20	59
SDS	0·001	No pentamidine added	99
		0	6
SDS	0·00067	No pentamidine added	76
		15	74

Marked protection against the inhibition of urease and invertase by Suramin was obtained, and in addition pentamidine was found to exert a powerful protective action against inhibition by other compounds of type *B*, e.g. Congo red and SDS (micelle). Although pentamidine was able to protect urease against Suramin it was not able to reverse the inhibition if it was added after Suramin had been in contact with the enzyme for an appreciable length of time.

Thus if pentamidine is added to the enzyme before, or only a few seconds after, the Suramin has been added, the full activity is maintained, but if addition of pentamidine is delayed, a slow inactivation occurs which is almost complete after 30 min. A similar result is obtained when SDS is used instead of Suramin (Table 3).

The effect of SDS on other enzymes

In addition to inhibiting urease, SDS was found to inhibit strongly several other enzymes, most of which had previously been found to be inhibited by Suramin (cf. Wills & Wormall, 1950*b*). The effect of SDS on different enzymes at different pH's is illustrated in Table 4. Invertase, malt α-amylase, tyrosinase and peroxidase each gave a sharply dropping pH/inhibition curve similar to that given by Suramin (Wills, 1952*a*). Other enzymes listed in Table 4 were inhibited over the whole pH range tested. Apart from carboxylase, which is powerfully inhibited by Suramin, enzymes which were not inhibited by 0·001 M-SDS were also those unaffected by Suramin.

In the case of urease the pH/inhibition curve for SDS was almost an exact replica of the curve for Suramin but with some other enzymes there was a tendency for the pH at which 50 % inhibition occurred to be shifted slightly to the alkaline side as compared with Suramin (Table 5).

Malt β-amylase which gave a sharp pH/inhibition curve with Suramin did not give a curve with 0·001 M-SDS, inhibition being maintained at about 85 % over the range pH 5·5–7·4.

The pH/inhibition curve for salivary amylase which was sharp in the case of Suramin, was smoother for SDS, shifted somewhat to the alkaline side and did not fall to zero inhibition even at alkaline pH's, e.g. 8·0 (Fig. 4).

Table 4. *Summary of the effect of* 0·001 M-*SDS on some enzymes*

The pH or pH range at which these enzyme tests were made was selected after a consideration of the optimum pH range of the enzyme, the stability of the enzyme at different pH's and previous experience of enzyme inhibition by Suramin.

Some enzymes inhibited by 0·001 M-SDS at the pH or pH range tested	pH
Malt β-amylase	5·0–7·0
Salivary amylase	5·0–7·8
Pancreatic lipase	6·0–8·0
Pancreatic esterase	6·8
Papain	5·0
Rennin	5·0
Succinic oxidase	7·4
Succinic dehydrogenase	7·4
D-Amino acid oxidase	8·0

Some enzymes unaffected by 0·001 M-SDS at, or near their optimum pH's	pH
Trypsin	7·4
Cytochrome oxidase	7·4
Carbonic anhydrase	6·8
Carboxylase	6·0
Serum cholinesterase	6·8
Papain	7·4

Some enzymes inhibited by 0·001 M-SDS below a critical pH	Critical pH
Urease	5·2
Invertase	4·5
Malt α-amylase	6·0
Tyrosinase	4·5
Peroxidase	5·0
Xanthine oxidase	6·5

Table 5. *Comparison of the effect of* pH *on the inhibition of some enzymes by* 0·001 M *Suramin and* 0·001 M-*SDS*

Enzyme	pH corresponding to 50 % inhibition: 0·001 M Suramin	0·001 M-SDS
Urease	5·1	5·1
Invertase	4·1	4·4
Malt α-amylase	5·0	5·6
Malt β-amylase	5·7	*
Tyrosinase	3·9	4·6
Peroxidase	4·9	4·8

* No quickly dropping curve obtained.

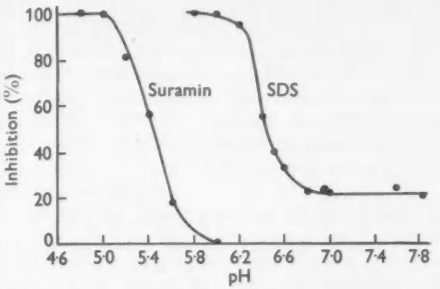


Fig. 4. Comparison of the effect of 0·001 M-SDS and 0·001 M Suramin on salivary amylase at different pH values.

The effect of SDS and bile salts on pancreatic lipase

For a considerable time there has been controversy concerning the exact mode of action of bile salts on pancreatic lipase, the question being whether bile salts directly stimulate lipase action or whether they act solely by a detergent action emulsifying the fatty substrate (cf. Willstätter, Waldschmidt-Leitz & Memmen, 1923; Willstätter & Memmen, 1924; Glick & King, 1932; Weinstein & Wynne, 1936; Holwerda, 1938). It was considered that it would be of interest to investigate the effect of SDS and related detergents on lipase with the object of ascertaining whether such compounds could replace bile salts in stimulating the action of the enzyme. If bile salts can be so replaced, this fact would strongly substantiate the hypothesis that they are mere emulsifying agents.

Many experiments were carried out using pig pancreatic lipase with various substrates at different pH's, and using different methods of estimation. The results obtained with any one method were consistent, but did not always agree with those obtained when another method of lipase estimation was used. By the use of the manometric method with 5% CO_2 and weak bicarbonate solution, and water-soluble triacetin as substrate so that no question of emulsification is involved, it was found that 0·001 M-SDS powerfully (92–75%) inhibits lipase over the pH range 6·0–8·0, no dependence of inhibition on pH being observable over this range. Sodium taurocholate (1%, w/v) also inhibited the reaction approximately 48% over the same pH range (Fig. 5) and 3% (v/v) Teepol completely inhibited triacetin hydrolysis at pH 7·4. These experiments do not substantiate the view that sodium taurocholate is an activator of lipase, at least so far as triacetin hydrolysis is concerned.

When triolein was used as substrate for the manometric method over the pH range 6·4–8·0 0·01 M-SDS again strongly inhibited the hydrolysis, and so did stronger SDS solutions (e.g. 0·1 M), but 1% (w/v) bile salt powerfully stimulated it. By the use of this substrate the effect of pH on the stimulating effect of bile salts on lipase activity was evident. Enzyme activity was weak in acid solutions (e.g. pH 6·4) and sodium taurocholate was able to stimulate the hydrolysis to a small extent only. The effect of the bile salt did, however, increase rapidly with rise of pH to a maximum at about pH 7·4 when a stimulation of 12 times the control rate was obtained (using 1% (w/v) sodium taurocholate) (Fig. 5).

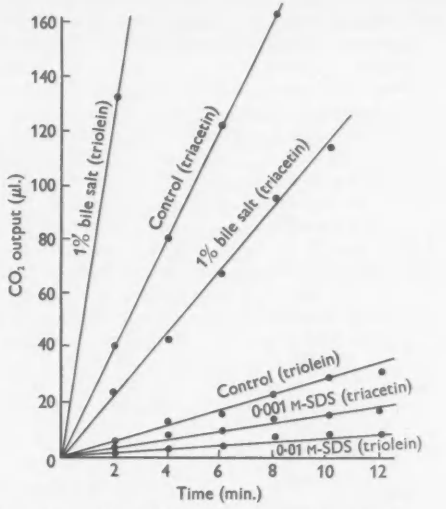


Fig. 5. Comparison of the effect of sodium taurocholate and SDS on pancreatic lipase using triacetin at pH 6·8 and triolein at pH 7·4.

All experiments with lipase so far described were carried out using 5% CO_2 and weak bicarbonate solutions. More acid conditions were better realized using 100% CO_2 and a stronger bicarbonate solution, and most experiments previously described were repeated under these conditions. The effects of sodium taurocholate and SDS were identical in strong and weak bicarbonate, but commercial Teepol in strong solutions (3–10%) was found markedly to stimulate triolein hydrolysis as much as 17 times the control rate at pH 7·6. This effect was sharply dependent on pH, for at pH 7·2 the same concentration of Teepol gave 100% inhibition of the hydrolysis. In weak bicarbonate solutions feeble stimulation (1·1 times the control) or no effect was observed. When Willstätter's method of estimating lipase action was used, Teepol powerfully inhibited the hydrolysis. It has not so far been possible to explain this difference, but it seems to be related in some way to the bicarbonate concentration

since with concentrations of bicarbonate between 0·02 and 0·3 M weak stimulation was observed.

Liquoide Roche or Stergene had little effect on the hydrolysis of either triacetin or triolein by lipase.

Using the continuous titration method for lipase and triacetin as substrate, SDS, in sharp contrast to the effect obtained manometrically, was found to stimulate the rate of hydrolysis to a small extent, 0·001 M-SDS producing about 1·5 times the rate of the control experiment. Sodium taurocholate (1 %, w/v) had little effect on the rate. Stronger solutions of SDS, such as 0·01 M, were a little more effective than 0·001 M and stimulated the rate to about twice that of the control, but the effect was negligible with solutions containing less than 0·00067 M-SDS.

Calcium ions powerfully stimulated the rate of triacetin hydrolysis by the lipase, a rate of 3·7 times that of the control being obtained with 0·01 M-Ca^{2+}. This stimulation was abolished by 0·01 M-SDS, an effect which may be due to precipitation of calcium dodecyl sulphate.

The effect of SDS on the succinic oxidase system

Various compounds have, from time to time, been discovered which (*a*) totally inhibit the complete succinic oxidase system, (*b*) have no effect on cytochrome oxidase, and (*c*) only partially inhibit succinic dehydrogenase. Bile salts, naphthoquinones and hydroxystilbene are some examples of such compounds which may be quoted (cf. Slater, 1949). The inhibition produced by such compounds is cited as evidence that there exist intermediate factors or enzymes other than succinic dehydrogenase, cytochrome *c*, and cytochrome oxidase which link succinate to oxygen.

SDS was found to belong to this class of compound (Rabinovitz & Boyer, 1950) and the present work supports these findings (Fig. 6). It was thought possible, however, that the weak inhibition (relative to that of the whole succinic oxidase system) obtained when investigating succinic dehydrogenase might be due to combination of the basic methylene blue (MB), with the acidic SDS, although Rabinovitz & Boyer (1950) rejected this view. This possibility was tested by examining the effect of 0·001 M-SDS alone and with the addition of MB in the same concentration as is used in succinic dehydrogenase experiments, on yeast fermentation. SDS (0·001 M) alone totally inhibited fermentation but addition of 0·08 % (w/v) MB reduced this inhibition almost to zero (Fig. 7). MB was also found to reduce SDS inhibition of urease and D-amino acid oxidase. These experiments lead to the conclusion that it is very likely that SDS actually does act on succinic dehydrogenase and that the relatively weak inhibition observed for succinic dehydrogenase is merely an artifact caused by combination of MB with SDS.

The effect of SDS on invertase

Invertase is completely (100 %) inhibited by 0·001 M-SDS at pH 4·0. In contrast to urease, inhibition does not fall rapidly with decreasing concentration of inhibitor, 0·0002 M-SDS giving nearly 100 % inhibition and 0·0001 M, 39 %.

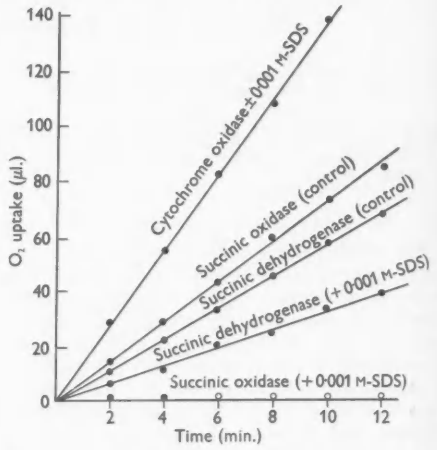


Fig. 6. Comparison of the effect of SDS on the succinic oxidase system, succinic dehydrogenase and cytochrome oxidase (0·001 M-SDS and 0·08 % methylene blue).

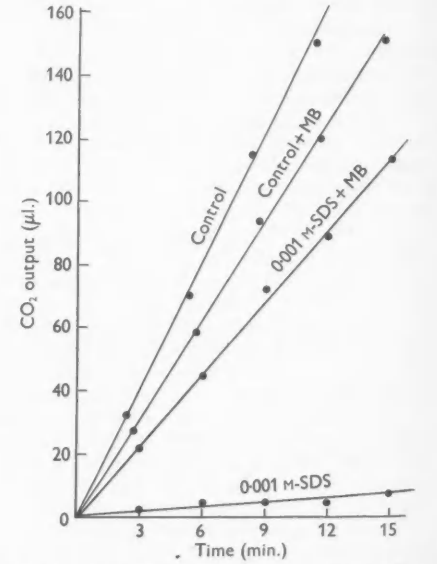


Fig. 7. Effect of 0·001 M-SDS and 0·08 % methylene blue on the fermentation of glucose by yeast.

Although a sharp pH/inhibition curve was obtained, inhibition by 0·001 M-SDS falling to zero at pH 4·7, the activity of the enzyme could not be regained by changing the pH from 4·0 (100 % inhibition) to pH 5·0 and was thus irreversible. In this respect invertase resembles urease, but the effect of SDS on invertase is in sharp contrast to the effect of Suramin which gives an inhibition which is reversible with pH.

The addition of the products of the hydrolysis, glucose and fructose, to invertase inhibited by 0·0002 M-SDS gave interesting results. Glucose produced no effect in any concentration but 0·067 M fructose practically overcame the inhibition and restored the activity (Fig. 8). Under the conditions of the experiment neither sugar had much effect on the rate of the control. Using 0·0002 M-SDS the effect of fructose fell off with a decrease in its concentration, a 0·01 M solution only producing a 12 % reduction of inhibition and the effect of 0·002 M was not detectable. The addition of a mixture of 0·04 M glucose and 0·04 M fructose to the inhibited enzyme, produced the same result as 0·04 M fructose alone. Galactose resembled glucose in having no effect on the inhibition.

The effect of SDS on milk-clotting enzymes

SDS, like Suramin, inhibited milk clotting by papain and rennin. SDS (0·005 M) completely inhibited in the case of both enzymes, whilst 0·001 M caused 50 % inhibition of the papain action and 65 % inhibition of rennin action. As in the case of Suramin it is believed that SDS combines with the substrate (casein) rather than with the enzyme.

The effect of SDS on trypsin

The effect of SDS on trypsin was investigated using a variety of substrates. Dilute solutions (0·001 M) of SDS had little or no effect with any substrate, but more interesting results were obtained when 0·01 M-SDS was used.

Casein. SDS (0·01 M) caused 10 % inhibition of the hydrolysis of a 5 % casein solution by trypsin.

Horse serum. The hydrolysis of the natural serum proteins by trypsin is very slow, but the reaction is strongly accelerated when the proteins are denatured (Haurowitz, Tunca, Schwerin & Goksu, 1945). Denaturation in the present series of experiments was normally accomplished by keeping the serum at 100° for 15 min. and then dispersing the coagulum with an equal volume of water in a Waring Blendor. SDS (0·01 M) was found to markedly speed up the rate of digestion of native serum but it could also accelerate, though to a smaller extent, the hydrolysis of denatured serum proteins (Fig. 9).

The effect of trypsin and 0·01 M-SDS on the insoluble denatured serum was to effect a rapid clarification of the solution which was complete after about 20 min. Neither trypsin nor SDS alone

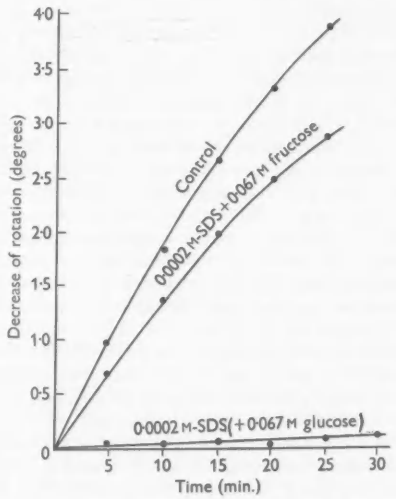


Fig. 8. Comparison of the effect of 0·067 M glucose and 0·067 M fructose on invertase inhibited by 0·0002 M-SDS at pH 4·0. The lowest curve represents results which were obtained with and without glucose.

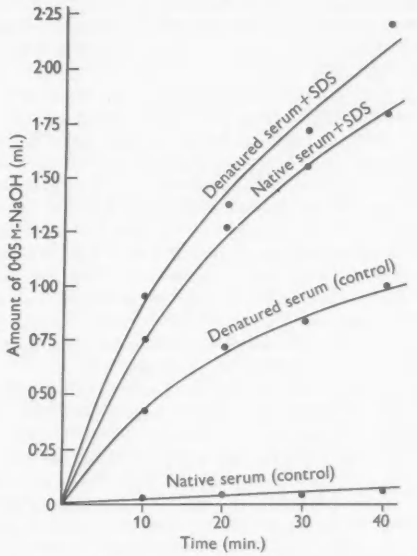


Fig. 9. Effect of 0·01 M-SDS on trypsin using native and denatured horse-serum proteins as substrates. Estimation by formol titration.

could effect this clarification even after 4 hr. The concentration of SDS used, 0·01 M, seemed to be about optimum under the conditions of these experiments, since stronger solutions (e.g. 0·02 M) did not give an enhanced effect and stimulation fell off rapidly with decreasing concentration and was negligible at 0·002 M. When the serum was denatured by treatment with acid the results obtained were practically identical with those obtained using heat-denatured serum.

SDS at 0·01 M was also found to accelerate the rate of tryptic digestion of horse-serum albumin, ovalbumin and to a small extent that of fibrin, but it had very little or no effect on the rate of digestion of these proteins when denatured.

DISCUSSION

The results of investigating the effect of a series of aromatic sulphonic acids on urease have, in general, supported the conception of a bridging mechanism of inhibition which was suggested as a possible explanation for the action of Suramin on urease (Wills & Wormall, 1950*b*). A consideration of Tables 1 and 2 will show that molecular size of the sulphonic acid cannot account for the inhibition produced. For example, the molecular weights of Congo red and Antrypol amine are very nearly the same, 696 and 701, respectively, and yet at pH 5·0 Congo red is at least 100 times more powerful an inhibitor than is the Antrypol amine. It is clear that the question of molecular size bears no relation to inhibition and some other explanation must be offered.

Type *B* compounds would probably combine with two adjacent or fairly close basic groupings of the enzyme in a manner similar to that suggested for Suramin by Wills & Wormall (1950*b*). These sulphonic acids, so combined with the enzyme, are likely to produce powerful steric blocking of the active centres and thus effect powerful inhibition. Inhibition of urease by type *B* compounds cannot be reversed by adjusting the pH or by adding pentamidine. This irreversibility suggests that binding of the enzyme's basic groups by this bridge type of combination causes distortion of the molecule and thus irreversible denaturation. The fact that SDS can act as a powerful protein-denaturing agent has been previously established (Putnam, 1948).

Type *A* sulphonic acids, on the other hand, would probably combine only at a single enzyme basic grouping. Results of experiments using type *A* compounds suggest that this type of combination offers little or no opposition to enzyme action and that there is little steric resistance to substrate molecules reaching the active centres. Furthermore, this combination does not strain the enzyme molecule since type *A* sulphonic acids appear to be easily liberated from the enzyme complex when the pH is made alkaline with respect to the isoelectric point of urease.

In dilute solutions, weaker than 0·001 M, the greater portion of the SDS molecules exist in solution as free ions which are weak inhibitors and may be classed with type *A* compounds. At a concentration of 0·001 M, and in stronger solutions, SDS takes on the micellar form, becomes a powerful inhibitor of urease and may be classed as type *B* if the $—SO_4$ groupings at the surface of the micelle are considered analogous to the spaced sulphonic acid groupings of type *B* sulphonic acids. Inhibition falls more rapidly with decreasing concentration in the case of SDS than with Suramin owing to the dissociation of the micelle into free ions. Dissociation cannot occur in the case of Suramin, but artificial dissociation of Suramin, i.e. the use of the half molecule, causes a sharp decline of inhibition (Tables 1 and 2). Experiments on reversibility of SDS inhibition support these views, since inhibition of urease by SDS solutions less concentrated than 0·001 M is reversible, whilst that of solutions more concentrated than 0·001 M is irreversible.

The biological action of a compound does not necessarily bear any relation to its action as an inhibitor of enzymes. For example, a list of a few compounds (Table 2) which behave in practically the same way towards urease, includes a dyestuff (Congo red), a trypanocidal drug (Suramin) and a detergent (SDS).

Pentamidine almost certainly protects urease against type *B* compounds by combining with the sulphonic acid groups to give a fairly stable, ring-like compound, bound by salt linkages. It might be regarded as a 'basic analogue' of type *B* compounds, and is the most powerful protective agent for enzymes against inhibition by type *B* compounds, some proteins excepted, that has so far been discovered. This fact suggests that its basic groupings may be a model of those found in the protein, and that the spacing of these basic groupings may closely resemble that in the enzyme proteins. One amidine grouping in a compound is not sufficient to protect enzymes against inhibition by type *B* compounds, since arginine will not protect urease against Suramin (Wills & Wormall, 1950*b*), and no protection is obtained using basic compounds such as adenine, guanine, histidine and lysine (Wills, unpublished work, 1950).

As in the case of experiments with Suramin, it has been found that urease is a useful model enzyme, and that many other enzymes resemble it in their reaction to SDS. In the many cases where the effect of SDS closely resembles that of Suramin it may be inferred that inhibition by these two substances occurs by a common mechanism, namely

the binding of acidic groups of the inhibitor with basic enzyme groups. In some cases, however, SDS is a more powerful inhibitor than is Suramin, especially with regard to its effect on certain enzymes on the alkaline side of their isoelectric points. The pH/inhibition curve for SDS does not always fall so sharply as in the case of Suramin and with some enzymes, e.g. α-amylase and salivary amylase, does not fall to zero inhibition at any pH, a fact which suggests that for these enzymes SDS inhibition may be more complex than is that of Suramin. A probable explanation of this difference is that in the case of SDS, in addition to simple ionic binding, there is the possibility of association of the long hydrocarbon chains with the peptide chains of the proteins. This is known to occur (cf. Putnam, 1948).

In the case of invertase, competition between the molecule of SDS and fructose suggests that each is attracted to a basic grouping, probably an amino grouping in the active centre of the enzyme. The view that fructose and acidic inhibitors were attracted to basic groupings in the active centre of the invertase was proposed by Quastel & Yates (1936) as a result of investigations on the effect of acid dyes on invertase. Their results, however, did not give such clear-cut evidence for the theory as do the SDS experiments described here. For example, they found that 0·22 M fructose reduced inhibition produced by $1{\cdot}19 \times 10^{-4}$ (w/v) Soluble blue from 66·5 to 52 %, and of $1{\cdot}14 \times 10^{-4}$ (w/v) Trypan red from 78·6 to 62·7 %, whereas this concentration of fructose reduced inhibition produced by 0·0002 M-SDS from 100 % to practically zero. Some experiments with Suramin gave results which were similar to those which Quastel & Yates (1936) obtained with acid dyes; for example 0·067 M fructose could only reduce the inhibitory effect of 0·001 M Suramin from 67 to 62 %. SDS thus appears to have a very specific affinity for certain basic groupings in the active centre of invertase which bind the fructose fragment of sucrose.

Attempts to replace bile salts as activators of lipase action with detergents were not generally successful. Although the detergent may be effective as an emulsifying agent, this action may be nullified by a direct inhibitory action on the enzyme. Nearly all the detergents tested did, however, inhibit the hydrolysis of triacetin by lipase. The stimulatory effect of strong Teepol solutions on the hydrolysis of triolein by lipase at pH 7·6 is not easy to explain and may be an artifact of the method of estimation. The dependence of the stimulation on the concentration of bicarbonate may be significant, however, especially in view of the fact that the bicarbonate concentration of the human duodenum may be as much as 0·1 M (Harrison, 1947). In the present series of experiments Teepol gave no stimulation of the rate of hydrolysis in 0·06 M-$NaHCO_3$ but powerfully stimulated in 0·28 M-$NaHCO_3$.

The effect of SDS on the hydrolysis of serum proteins by trypsin may be readily explained, since it is known that SDS is a powerful denaturing agent (Putnam, 1948) and also that it stimulates acid hydrolysis of proteins. Trypsin, in general, hydrolyses denatured proteins much more rapidly than native proteins (Haurowitz *et al.* 1945) and SDS greatly accelerates protein hydrolysis by its denaturating action. It is probable that, in addition, SDS has some direct effect on the peptide linkage of proteins since it accelerates acid hydrolysis (Steinhardt, 1941), and actual combination of SDS with peptide linkages may occur after saturation of the basic groups of the protein.

To summarize, the results of the investigations on the effect of SDS on enzymes are in good agreement with those obtained from the study of the combination of SDS with inert proteins, many results of which are summarized by Putnam (1948). Enzymes for the most part do not appear to react with these anionic detergents in a very different manner from other proteins, but a study of enzyme inhibition can often give a more sensitive and precise means of studying detergent-protein combination than can conventional methods using inert proteins.

SUMMARY

1. The effect of several aromatic sulphonic acids on urease has been studied at pH 5·0, and, depending as to whether they are powerful or weak inhibitors they have been divided into two groups. Inhibition has been related to the structure of the sulphonic acids.

2 Sodium dodecyl sulphate (SDS) (0·001 M) completely inhibits urease at pH 5·0, but inhibition falls off sharply with increased alkalinity and decreasing concentration of SDS. Inhibition is irreversible with 0·001 M but reversible with 0·0005 M-SDS.

3. Commercial detergents Teepol and Liquoide Roche act on urease in a similar manner to SDS, but sodium taurocholate is only a weak inhibitor.

4. Pentamidine protects urease against inhibition by Suramin, Congo red and SDS at pH 5·0 but it does not reverse inhibition if these compounds have prior contact with the enzyme.

5. Most enzymes tested are inhibited by SDS if the pH is suitably adjusted. Many give sharp pH/inhibition curves similar to those given by Suramin, but the pH of 50 % inhibition is sometimes more alkaline than that obtained with Suramin.

6. Sodium taurocholate, SDS and Teepol inhibit the hydrolysis of triacetin by pancreatic lipase. Sodium taurocholate stimulates, but SDS inhibits triolein hydrolysis. Under certain conditions Teepol stimulates triolein hydrolysis.

7. SDS (0·001 M) inhibits succinic dehydrogenase to a less extent than it does the whole succinic oxidase system, possibly owing to its combination with MB. SDS has no effect on cytochrome oxidase.

8. SDS (0·0002 M) strongly inhibits invertase at pH 4·0. This inhibition can be completely abolished by 0·067 M fructose but not by glucose.

9. SDS has little effect on the hydrolysis of casein by trypsin, but it strongly stimulates digestion of native serum proteins and to a less degree that of the denatured proteins.

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Dietary Fat and Body Fat: the Composition of the Back Fats of Pigs Fed on a Diet Rich in Cod-liver Oil and Lard

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In a recent study (Garton, Hilditch & Meara, 1952) of the depot fats of a pig fed on a diet rich in whale oil it was found that apparently unchanged whale-oil glycerides were present in the depots. However, since the whale oil was ingested in very large amounts and represented practically all the fat in the diet, the depot glycerides which resembled whale oil would have been substantially the same whether they had been hydrolysed and resynthesized during passage from the intestine to the lymphatics or whether they had been absorbed intact. That the pig is capable of depositing dietary fatty acids in its fat depots is well known (for a review see Hilditch, 1947, pp. 99–101), though it is not known to what extent dietary triglycerides are absorbed as such and deposited in the fat depots.

The present study was undertaken to obtain evidence in this direction by feeding a diet containing a mixture of lard and cod-liver oil to two young pigs and then studying the composition of the resultant depot fats. If intestinal hydrolysis occurred to any appreciable extent, followed by resynthesis in the intestinal wall or elsewhere, the component fatty acids characteristic of cod-liver oil (especially the polyethenoid C_{20}–C_{22} acids) and of lard would mix and result in the formation of depot glycerides which would not be separable by low-temperature crystallization into groups of glycerides resembling lard and cod-liver oil, respectively. As described below, such a separation was, however, effected, although the cod-liver oil had undergone oxidative changes, and it seems therefore that

intestinal hydrolysis of the ingested fats with subsequent resynthesis of 'new' glycerides had not taken place to any appreciable extent.

METHODS AND RESULTS

Animals and diet

Two normal Large White × Wessex litter-mate piglets (1 gilt and 1 hog) were fed from weaning at 8 weeks on a diet containing commercial lard and cod-liver oil as a mixture of equal parts by weight; the analytical values of the two fats are given in Table 1.

The amount of fat mixture was gradually increased until at the beginning of the seventh week of the experiment it comprised 50% by weight of the diet. Full details of the diet, feeding scale, management of the animals and their weight gains have been given by Garton & Naftalin (1953).

No supplementary antioxidant was included in the diet since Walley (1952), at the Animal Research Station, School of Agriculture, Cambridge, found that pigs could be raised to bacon weight (200 lb.) on a diet containing 50% crude whale oil without added vitamin E. The mean unsaturation of the C_{20} and C_{22} polyethenoid fatty acids of cod-liver oil and whale oil is of the same order (see Hilditch, 1947), and it was calculated that the amounts of these acids in a diet containing 25% of cod-liver oil would be roughly the same as that furnished by 50% of whale oil. However, during the seventh week of the present experiment, the animals suddenly lost their appetites and became oedematous and unsteady in their gait. They were killed at the end of the seventh week and examined *post mortem*. The morbid anatomical findings, which resembled the exudative condition induced in chickens fed on high-fat diets deficient in vitamin E (Dam, 1944*a*, *b*), have already been described in detail (Garton & Naftalin, 1953).

Extraction of back fats and their characteristics

As much as possible of the inner- and outer-back adipose tissue was removed from each animal; there was an almost complete absence of perinephric fat. The tissues, which were stored at 2° overnight, were cut into small pieces and exhaustively extracted with boiling acetone. The acetone-soluble material was extracted with light petroleum (b.p. 40–60°) and the lipid material from each tissue was separately recovered and found to have the analytical characteristics shown in Table 2.

All four fats were dark brown and semi-solid at room temperature; they had a green fluorescence in daylight and an odour of cod-liver oil.

Table 1. *Analytical characteristics of lard and cod-liver oil as fed to pigs*

	Lard	Cod-liver oil
Iodine value	60·4	185·1
Saponification equivalent	284·5	301·3
Peroxide value (ml. 0·002 N-$Na_2S_2O_3$/g.)	1·0	11·7
Free fatty acid (as oleic, %)	0·25	0·04
Unsaponifiable matter (%)	0·80	1·80

Separation of the component glycerides of the back fats of the hog

The characteristics given in Table 2 showed that all the fats were considerably peroxidized, which would probably render the determination of their polyethenoid acid content very difficult. Nevertheless, it was decided to attempt a separation, by low-temperature crystallization, of the glycerides of the back fats of one animal. Preliminary experiments showed that mixtures of cod-liver oil and lard could be effectively resolved by low-temperature crystallization. Mixtures of iodine value 80–90 were separated almost completely, as judged by analytical results (iodine value and saponification equivalent), into their two components by successive crystallizations at −40° of the lard glycerides from a 10% solution of the mixture in acetone.

A quantity of each of the inner- and outer-back fats of the hog was therefore crystallized from 10% solution in acetone at −40°, the deposited (more saturated) glyceride being recrystallized under the same conditions and the procedure repeated until only a small amount of glycerides remained in solution. In this way the fractions shown in Table 3 were obtained.

The respective portions left in solution from five crystallizations were pooled and so from each fat two groups of glycerides were obtained, the characteristics of which are given in Table 4.

Separation of the component fatty acids of the back fats of the hog

Each group of glycerides was saponified and the fatty acids removed by extraction with peroxide-free ether. The acids were then crystallized for 4–5 hr. from 10% solution in acetone or peroxide-free ether. The results of the solvent separations are shown in Table 5.

Fractionation of the methyl esters of the fatty acids

Each group of fatty acids derived by low-temperature crystallization was converted into methyl esters by refluxing with methanol containing 1% H_2SO_4. The esters were

Table 2. *Analytical characteristics of inner- and outer-back fats*

	Source of fat			
	Hog		Gilt	
	Inner back (348 g.)	Outer back (482 g.)	Inner back (239 g.)	Outer back (333 g.)
Iodine value	81·8	86·8	84·5	85·5
Saponification equivalent	277·8	282·0	278·7	278·9
Peroxide value*	25·3	34·1	55·0	28·3
Free fatty acid (as oleic, %)	1·2	1·1	1·4	1·1
Unsaponifiable matter (%)	0·84	0·40	0·44	0·44

* Determined immediately after removal of extraction solvents.

washed with 1% (w/v) Na_2CO_3, dried and fractionally distilled at approximately 0·1 mm. pressure through an electrically heated column. The methods employed in the distillation and for the analytical (including spectrophotometric) examination of the ester fractions were those described by Gupta & Hilditch (1951). The composition of each ester fraction was then calculated according to the methods given by Hilditch (1947, pp. 498–510) and Gupta & Hilditch (1951), and therefrom the percentage composition (by weight) of each group of acids from each fat was derived.

(*a*) *Outer-back fat.* The distillation residues of the methyl esters of the acids of group *D* (Table 5) amounted to 15% and showed a fall from an iodine value of 286·4 (last distilled ester fraction) to 139·7 for the residual esters freed from unsaponifiable matter. It was therefore apparent that destruction of polyethenoid acids had taken place to a very considerable extent and that calculation of the fatty acid composition of this fraction (and hence of the whole group of acetone-soluble glycerides) was not possible. Accordingly, the fatty acids of groups *A*, *B* and *C* of this fat were not submitted to ester fractionation.

(*b*) *Inner-back fat.* It was possible in this case to compute the fatty acid composition of each ester fraction and hence of each group of glycerides, the composition of which is given in Table 6. The composition of the residual esters of the fatty acids of group *E* (Table 5), after removal of unsaponifiable matter, was calculated as described by Hilditch (1947, p. 509), thus showing acids of somewhat higher unsaturation than were present in the fat as analysed.

Table 3. *Segregation of back-fat glycerides of hog by successive crystallization from 10% solution in acetone at −40°*

The weights of material refer to the fat remaining in solution, except for the last figure in each 'weight' column, which refers to the insoluble glycerides.

	Outer-back fat (245·0 g.)		Inner-back fat (227·6 g.)	
	Weight (g.)	Iodine value	Weight (g.)	Iodine value
First crystallization	46·0	150·7	41·9	133·0
Second crystallization	19·3	126·7	15·7	116·6
Third crystallization	5·2	111·1	6·6	108·8
Fourth crystallization	4·3	103·0	5·3	99·6
Fifth crystallization	4·0	99·1	4·5	98·5
Glycerides deposited (fifth crystallization)	166·2	64·0	153·6	60·3

Table 4. *Groups of glycerides separated from hog back fats*

	Outer-back fat			Inner-back fat		
	Weight		Iodine value	Weight		Iodine value
	(g.)	(% of total)		(g.)	(% of total)	
Glycerides insoluble in acetone at −40°	166·2	67·8	64·0	153·6	67·5	60·3
Glycerides soluble in acetone at −40°	78·8	32·2	137·0	74·0	32·5	126·5

Table 5. *Low-temperature crystallization of the mixed acids from each group of hog back-fat glycerides*

Fraction	Conditions	Weight (g.)	Weight (% of total)	Iodine value
	Outer-back fat			
(1) Acids from glycerides insoluble in acetone at −40°				
A	Insoluble in ether at −35°	67·4	46·8	24·6
B	Soluble in ether at −35°	76·5	53·2	105·1
(2) Acids from glycerides soluble in acetone at −40°				
C	Insoluble in acetone at −40°	21·7	31·7	35·3
D	Soluble in acetone at −40°	46·7	68·3	169·0
	Inner-back fat			
(1) Acids from glycerides insoluble in acetone at −40°				
A	Insoluble in ether at −30°	43·1	31·6	3·4
B	Soluble in ether at −30°	38·3	28·0	61·0
C	Soluble in acetone at −35°	55·2	40·4	109·5
(2) Acids from glycerides soluble in acetone at −40°				
D	Insoluble in acetone at −45°	30·2	45·4	70·9
E	Soluble in acetone at −45°	36·3	54·6	179·5

Table 6. *Component acids of solvent-separated fractions from each group of glycerides of the hog inner-back fat*

Separation into fractions *A*, *B*, *C*, *D* and *E* is shown in Table 5.

Acids from glycerides insoluble in acetone at −40°

Acid	Fraction *A* (%)*	Fraction *B* (%)*	Fraction *C* (%)*	Fatty acids in whole group of glycerides (excluding unsaponifiable matter) (%, w/w)	(%, by mol.)
Myristic	—	0·25	0·29	0·5	0·6
Palmitic	22·99	7·57	1·58	32·2	34·3
Stearic	7·41	1·59	—	9·0	8·6
Arachidic	0·18	—	—	0·2	0·2
Unsaturated C_{14}	—	0·06	0·45	0·5	0·6
Unsaturated C_{16}	0·05	1·20	2·70	4·0	4·2
Unsaturated C_{18}	0·97	17·23	30·18	48·4	46·9
Unsaturated C_{20}	—	—	5·20	5·2	4·6
Unsaponifiable	—	0·10	—	—	—
Mean unsaturation† of:					
C_{14} acids	—	−2·0	−2·0	−2·0	
C_{16} acids	−2·0	−2·0	−2·4	−2·3	
C_{18} acids	−2·0	−2·0	−2·6	−2·4	
C_{20} acids	—	—	−4·0	−4·0	

Acids from glycerides soluble in acetone at −40°

Acid	Fraction *D* (%)*	Fraction *E* (%)*	Fatty acids in whole group of glycerides (excluding unsaponifiable matter) (%, w/w)	(%, by mol.)
Myristic	—	0·38	0·4	0·5
Palmitic	12·94	1·80	14·9	16·6
Stearic	2·36	—	2·4	2·4
Unsaturated C_{14}	—	1·34	1·3	1·6
Unsaturated C_{16}	9·08	8·60	17·9	19·0
Unsaturated C_{18}	19·02	24·42	43·9	42·3
Unsaturated C_{20}	2·00	12·51	14·7	13·7
Unsaturated C_{22}	—	4·48	4·5	3·9
Unsaponifiable	—	1·07	—	—
Mean unsaturation† of:				
C_{14} acids	—	−2·0	−2·0	
C_{16} acids	−2·0	−2·9	−2·4	
C_{18} acids	−2·3	−3·5	−3·0	
C_{20} acids	−2·0	−7·0	−6·3	
C_{22} acids	—	−10·3	−10·3	

* Component acids as % (w/w) of each solvent-separated fraction.
† Mean unsaturation expressed as number of H atoms required to convert 1 mol. of acid into the saturated state.

Table 7. *Separation of mixed fatty acids of lard and cod-liver oil by low-temperature crystallization*

Solubility characteristics of fraction	Weight (g.)	Weight (% of total)	Iodine value
	Lard		
A Insoluble in ether at −30°	93·1	44·3	9·7
B Soluble in ether at −30°	78·7	37·4	82·1
C Soluble in acetone at −60°	38·5	18·3	145·0
	Cod-liver oil		
A Insoluble in ether at −40°	44·6	18·5	26·1
B Soluble in ether at −40°	100·4	41·8	154·1
C Soluble in acetone at −60°	95·5	39·7	286·4

Fatty-acid composition of lard and cod-liver oil

The component fatty acids of the lard and cod-liver oil which were fed to the animals were determined in a similar manner to that already described for the back fats. Preliminary resolution of the mixed fatty acids was carried out by low-temperature crystallization to give the groups shown in Table 7. The final results of the component acid analyses of the two fats are summarized in Table 8.

DISCUSSION

It is apparent from Table 2 that all four back fats are essentially similar, with the outer-back fat in each case showing, as might be expected, a slightly higher degree of unsaturation. That the fats have undergone oxidation to a considerable extent is shown by their high peroxide values and their low saponification equivalents. The saponification equivalents might have been expected to be intermediate between those of the lard and cod-liver oil which were administered, but in each case the equivalent is even lower than that of the lard, indicating that short-chain acids, derived by oxidative breakdown, are present. However, the saponification equivalent of the hog inner-back fat, when calculated from the determined composition of the two groups of acetone-separated glycerides together with the unsaponifiable matter, gives a value 288·7 which is 10·9 units higher than that found directly on the original back fat (277·8). When the mixed fatty acids were washed with water it was found that repeated extraction failed to remove completely all traces of acidity, indicating a slow removal of acidic products from the mixture.

Successive crystallizations of the hog inner- and outer-back-fat glycerides from acetone at $-40°$ (Tables 3 and 4) resolved each fat into two groups of glycerides in the proportions of two parts by weight of a fat the iodine value of which approximated closely to that of the lard as fed, to one part of a fatty oil of much greater mean unsaturation. Detailed examination (Table 6) of the fatty acid composition of the acetone-insoluble glycerides of the hog inner-back fat revealed a marked resemblance to that of the lard (Table 8), both in respect of the relative amounts of each acid present and the degree of unsaturation of the C_{14}–C_{18} acids.

Any fat synthesized *de novo* by the animals from carbohydrate and laid down in the depots would be indistinguishable from the lard which was fed (cf. the composition of the inner-back fat of a pig fed on a low-fat diet (Table 8)). Complete separation of lard and biosynthesized back-fat glycerides from cod-liver-oil glycerides by the physical process of crystallization cannot be achieved and the increased content of C_{20} acids (characteristic of cod-liver oil) of a fairly low degree of unsaturation in the acetone-insoluble glycerides is therefore not surprising.

If, in fact, cod-liver-oil glycerides had been absorbed intact, the fatty-acid composition of the acetone-soluble glycerides of the hog inner-back fat would not be expected to reveal a very close resemblance to that of the oil as fed, since it was evident that the back fat was oxidized to a considerable extent; such oxidation would involve loss of unsaturated (polyethenoid) acids. Nevertheless, it is evident from Table 8 that the acetone-soluble glycerides resemble cod-liver oil very strikingly as far as their content of saturated fatty acids is concerned, and that, although the amounts of polyethenoid acids present are much lower than in cod-liver oil, they exhibit unsaturation of a similar high order.

Table 8. *Component acids (%, w/w) of inner-back solvent-separated glycerides and of lard and cod-liver oil, together with component acids of inner-back fat of pig on low-fat diet*

			Hog inner-back fat		
Acid	Inner-back fat (low-fat diet) (Hilditch & Pedelty, 1940)	Lard (present work)	Glycerides insoluble in acetone at $-40°$ (present work)	Glycerides soluble in acetone at $-40°$ (present work)	Cod-liver oil (present work)
Myristic	0·9	0·5	0·5	0·4	1·1
Palmitic	27·5	27·1	32·2	14·9	13·8
Stearic	15·1	15·6	9·0	2·4	2·7
Arachidic	—	0·7	0·2	—	—
Unsaturated C_{14}	0·2	0·4	0·5	1·3	0·4
Unsaturated C_{16}	1·7	2·8	4·0	17·9	8·0
Unsaturated C_{18}	51·5	51·9	48·4	43·9	27·3
Unsaturated C_{20}	3·1	1·0	5·2	14·7	29·4
Unsaturated C_{22}			—	4·5	17·3
Mean unsaturation of					
C_{14} acids	−2·0	−2·0	−2·0	−2·0	−2·0
C_{16} acids	−2·0	−2·1	−2·3	−2·4	−2·3
C_{18} acids	−2·3	−2·3	−2·4	−3·0	−3·3
C_{20} acids	−5·0	−2·3	−4·0	−6·3	−6·3
C_{22} acids			—	−10·3	−7·9

The total percentage of unsaturated fatty acids in the acetone-soluble glycerides (82·3%) is the same as the corresponding figure (82·2%) for the cod-liver oil itself, and it is therefore suggested that oxidative splitting of the C_{20} and C_{22} polyethenoid acids occurred giving rise to increased amounts of C_{18} and C_{16} unsaturated acids. We consider that the cod-liver oil was absorbed essentially unchanged from the intestine and laid down in the depots additively along with lard and any fat synthesized by the animal from carbohydrate.

The much discussed question of the mechanism of fat absorption has recently been reviewed by Frazer (1952), who concludes that the intraluminar phase consists of emulsification of triglycerides to yield fine particles (diam. $< 0{\cdot}05\ \mu$.) which are then readily absorbed. Emulsification requires a triple combination of fatty acids, partially hydrolysed triglyceride and bile salt, involving a certain degree of lipolysis by pancreatic lipase. While the present work supports the view that glycerides can be absorbed in the unhydrolysed state, it gives no indication of the extent of hydrolysis, though it is evident that it did not take place to any great extent, since the triglycerides which appeared in the depots were identified as a mixture of typical pig fat (lard) and cod-liver oil in an oxidized form.

Aspects of the present study are of interest in relation to the well-known injurious effects of feeding unsaturated fat in a diet low or deficient in vitamin E (see review by Blaxter & Brown, 1952). Manifestations resulting from such a diet vary among species, but include muscular dystrophy, encephalomalacia, oedema and brown coloration of the depot fats; it appears that these conditions are related to the content of unsaturated (mainly polyethenoid) fatty acids in the diet (cf. Dam & Granados, 1945). When the available resources of antioxidant are depleted, oxidation of polyethenoid acids proceeds unchecked, as apparently occurred in the present experiment. It may well be that the lesions produced result from the liberation of plasma-soluble oxidation products (especially from fat depots) which then interfere with normal metabolism in other parts of the body.

That pigs can be raised to bacon weight (200 lb.) on a diet containing 50% of crude whale oil with no overt clinical signs of antioxidant depletion (Walley, 1952) may mean that the polyethenoid acids of this oil are less prone to oxidative attack by virtue of their chemical structure or that the oil itself contains considerably more vitamin E than does cod-liver oil which, according to Brown (1953), contains about 30 mg. α-tocopherol/100 g.

SUMMARY

1. The inner- and outer-back fats of two pigs fed on a diet containing fat (as a mixture of equal parts of lard and cod-liver oil) have been examined. The fats were dark brown and oxidized to a considerable degree.

2. Crystallization of the back fats of one animal from acetone at $-40°$ separated each into 68% of an insoluble glyceride fraction, the iodine value of which approximated to that of lard, and 32% of soluble glycerides of much higher mean unsaturation.

3. Determination of the component fatty acids of the acetone-separated glycerides from the inner-back fat showed that the insoluble glycerides resembled normal pig fat except for the presence of somewhat more unsaturated acids of the C_{16} and C_{20} series than are usually present in the latter. The acetone-soluble glycerides showed a striking resemblance to cod-liver oil in as far as their content of saturated fatty acids was concerned, but contained less polyethenoid C_{20} and C_{22} fatty acids with correspondingly increased amounts of C_{16} and C_{18} unsaturated acids.

4. It was concluded that the lard and cod-liver oil were absorbed essentially unchanged and deposited additively along with typical 'synthesized' pig fat in the depots; it is suggested that depletion of anti-oxidant (vitamin E) then occasioned oxidation of the polyethenoid fatty acids of the cod-liver oil.

5. The results are discussed in relation to the injurious effects of diets containing unsaturated fat and little or no vitamin E.

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Comparative Studies of 'Bile Salts'

8. PRELIMINARY EXAMINATION OF BILE SALTS BY PAPER CHROMATOGRAPHY

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Methods for the examination of free bile acids have been greatly improved in recent times and progress in this respect can be said to be satisfactory (see, for example, Bergström & Sjövall, 1951; Sjövall, 1953; Wootton, 1953).

However, until very recently no real advance was made in methods for the study of the native bile salts themselves. Ahrens & Craig (1952) showed that counter-current distribution could be applied with success to ox bile salts, and they published the first comprehensive analysis of this material. Subsequently, Sjövall (1952; 1954) demonstrated that paper-partition chromatography could be used for the same purpose. Bergström & Norman (1953*a*) and Norman (1953) used partition chromatography for the separation of bile acids as they occur in bile and faeces. Bile acids conjugated with taurine could be separated as a group, and free bile acids or those conjugated with glycine could be separated as individual compounds by the use of different solvent systems.

The present report is of work designed to investigate the application of the paper chromatography of Sjövall (1952, 1954) to biological problems.

It was decided to investigate especially (*a*) the occurrence of bile acids conjugated with glycine in vertebrates other than mammals and (*b*) species known or thought to have remarkable bile salts.

Systematic names for free bile acids are as follows: cholic acid (3α:7α:12α-trihydroxycholanic acid); pythocholic acid (probably 3α:12α:16α-trihydroxycholanic acid); deoxycholic acid (3α:12α-dihydroxycholanic acid); chenodeoxycholic acid (3α:7α-dihydroxycholanic acid); hyodeoxycholic acid (3α:6α-dihydroxycholanic acid); ursodeoxycholic acid (3α:7β-dihydroxycholanic acid); trihydroxycoprostanic acid (3α:7α:12α-trihydroxycoprostanic acid). Glycine and taurine conjugates are indicated by the prefixes 'glyco' and 'tauro'.

METHODS

Paper chromatography (see Sjövall, 1952, 1954). This was carried out using systems as follows: Glyco system, G_1: mobile phase, 75% (v/v) *iso*propylether/*n*-heptane; stationary phase, 70% (v/v) acetic acid/water. Glyco system, G_2: mobile phase, 85% (v/v) *iso*propyl ether/*n*-heptane; stationary phase, as G_1. Glyco system, G_3: mobile phase, 60% (v/v) *iso*propyl ether/*n*-heptane; stationary phase, as G_1. Tauro system, *T*: mobile phase, *n*-butanol equilibrated with 3% (v/v) acetic acid/water; stationary phase, 70% (v/v) acetic acid/water.

The mobile and stationary phases of G_1, G_2 and G_3 were equilibrated by shaking and separating at room temperature; such equilibration cannot be carried out with system *T*. Whatman's no. 3MM paper was prepared for all systems by washing with 70% (v/v) acetic acid/water and heating until superficially 'dry' in an oven (about 5 min. at 90°). The spots of bile salt solutions were then applied at the starting line (about 10 cm. from one end of the paper) in drops, the approximate volume (0·008 ml.) of which had been previously determined by weighing. Hence, it was possible to calculate the figures, given in Table 1, for μg. of bile salt put on to the starting line.

Chromatography (ascending) with glyco systems G_1 and G_2. A strip of paper (about 12 × 40 cm.) treated and loaded with bile salts as above was hung (with the starting line at the bottom) in a glass tank tilted so that mobile phase on the floor of the tank was out of contact with the paper. For equilibration, wide strips of Whatman's no. 1 paper moistened with and dipping into the mobile phase were placed in the tank around the walls, and other paper strips were dipped into and left saturated with stationary phase, contained in a beaker also in the tank. The tank was sealed first with a cloth wetted with mobile phase, then a cellophan cover and finally with a rubber cover. After about 12–16 hr. at about 20–23°, when equilibration was presumed to be complete, the tank was tilted so that the mobile phase ran on to the bottom of the paper. Ascension of the solvent front to about 20 cm. above the starting line took 2–4 hr. and the paper was then removed and dried at 90–100°. It was sprayed with 10% (w/v) phosphomolybdic acid in ethanol (Kritchevsky & Kirk, 1952) and heated at about 100° until the blue spots developed (about 5 min.).

Chromatography (descending) with tauro system T and glyco system G_3. The paper, treated and loaded with bile salts as described above, was hung (with the starting line at the top) from a trough in a glass tank so that the starting line was about 10 cm. from the trough and the bottom of the paper clear of the floor of the tank. Mobile phase covered the floor of the tank and also saturated strips of Whatman's no. 1 paper on the walls. Stationary phase in a beaker in the tank ran up a paper strip. The tank was sealed with a cloth, cellophan and then a rubber cap. After about 16 hr., these were pierced with a hypodermic syringe and mobile phase was thus injected into the trough. The liquid was allowed to run down the paper for a total distance of about 30 cm.

When G_3 was used, the chromatogram was run for 18 hr., the mobile phase dripping off the lower edge of the paper. The paper was dried and sprayed as described above.

Standard solutions. Determination of R_F values was of little use in the present work; the values varied considerably with temperature and equilibration conditions, and this is perhaps not surprising in view of the method used to establish a stationary phase in the paper (see above). However, reliable and consistent results were obtained by comparison with standard bile salt preparations run on the same paper. Sodium salts of taurocholic, taurodeoxycholic, glycocholic and glycodeoxycholic acids were prepared as described by Cortese (1937) and purified by passage through the ion-exchange resin Amberlite IRA-400 (OH) (Rohm and Haas Co., Philadelphia, U.S.A.), which removed inorganic impurities. Glyco- and tauro-chenodeoxycholic acids were prepared as described by Bergström & Norman (1953*b*). Examination of the chromatograms suggested that the cholic acid derivatives might have contained a little free cholic acid. For the present work 'synthetic' conjugated hyodeoxycholic acids were not available, and the situation with respect to the behaviour on paper of these substances is therefore not quite clear (see Discussion). Taurine conjugates of deoxycholic and of chenodeoxycholic acids ran at the same rate with system *T*. With systems G_1 and G_2, glycodeoxycholic and glycochenodeoxycholic acids ran at closely similar rates.

Standard solutions contained 2–5 mg./ml. in water (cholic acid in ethanol). With the spraying reagent used it was possible to detect about 0·8 μg. of glycocholate and 1·6 μg. of taurocholate, and these figures were used as the basis for the calculation of columns 3 and 4 in Table 1.

Preparation of bile salts. It was found, after preliminary trials, that crude bile salts as prepared by Haslewood & Wootton (1950) could be directly dissolved in water and applied to the papers. This procedure resulted in some streakiness, due probably to impurities in the bile salts, but this did not impair the visibility of the spots. The use of crude bile salts greatly shortened the time needed for analysis.

RESULTS

Examination for glycine conjugation. Examples of papers obtained were photographed and are shown in Figs 1 and 2. Table 1 indicates that in the bile salts of seven species of fish studied, glycocholic acid could not have constituted more than about 0·7 % and glycodeoxycholic acid not more than about 1·3 %. The corresponding figures for eight species of birds were 0·8 and 1·6 %. Glycine conjugates were not detected in the chicken, dogfish or frog (Fig. 2) and probably not in the wallaby (Fig. 1); they could be found in the goat and whale (Fig. 1).

The significance of these findings is more fully discussed below.

Examination of various bile salts. These can be divided into groups, as shown in Table 2, according to whether they are known or expected to contain

Table 1. *Examination of the bile salts of fish and birds for glycocholic or glycodeoxycholic acids by paper chromatography*

Details of the method are described in text.

Species	Approx. concentration of bile salts (mg./ml.)	Approx. wt. put on start line (μg.)	Minimum amount of glycocholic acid detectable (%)	Minimum amount of a glycodeoxycholic acid detectable (%)	Notes on bile
Cod, *Gadus morrhus*	28·7	229	0·3	0·7	Contains cholic acid (Haslewood & Wootton, 1950)
Plaice, *Pleuronectes platessa*	17·1	137	0·6	1·2	As above
Flounder, *P. flesus*	16·1	129	0·6	1·2	
Bass, *Morone labrax*	17·8	142	0·6	1·1	Expected to contain taurocholate
Brown trout, *Salmo trutta*	14·9	119	0·7	1·3	
Rainbow trout, *S. irideus*	19·9	159	0·5	1·0	
Dogfish, *Acanthias vulgaris*	19·4	155	0·5	1·0	Contains scymnol, or a precursor, conjugated with sulphate (Cook, 1941)
Domestic chicken	17·9	143	0·6	1·1	
Golden eagle, *Aquila chrysaëtus*	21·2	169	0·5	0·9	
Bateleur eagle, *Terathopius ecaudatus*	21·5	172	0·5	0·9	
Eagle Owl, *Bubo bubo*	17·8	142	0·6	1·1	
Shoebill, *Balaeniceps rex*	12·8	102	0·8	1·6	See Discussion
Cassowary, *Casuarius rogersi*	15·0	120	0·7	1·3	
King penguin, *Aptenodites patogonica*	21·0	168	0·5	0·9	
Black-thighed Hornbill, *Bycanistes cylindricus*	17·3	138	0·6	1·2	

Table 2. *Results of examination by paper chromatography of bile salts of various species*

References to Table 1 are to Latin names, concentrations and notes on bile. Abbreviations: *M*, modern; *P*, primitive; *MP*, most primitive; G_3, *T*, glycine, taurine systems, respectively; *TC*, spot as taurocholate; *TD*, spot as taurodeoxycholate.

Species (The figures in parentheses refer to the approx. concentration of bile salts in mg./ml. used for the experiment)	Notes on bile salts	System	References to figures	Results*
Pig (15·3); Coypu, *Myocastor coypus* (11·3)	*M*, see Discussion	G_3 & *T*	3	*TD* (on starting line in Fig. 3) and see Discussion
Dog, *Canis familiaris* (19·4)	*M*	*T*	—	*TC* and *TD*
Wallaby, *Macropus ruficollis* (20·8); domestic goat (21·0); fin whale, *Balaenoptera physalus* (17·4); polar bear, *Thalarctos maritimus* (18·4)	All *M*	*T*	—	*TC* and *TD*
Himalayan bear, *Ursus tibetanus* (21·3)	*M*, possibly contains ursodeoxycholate	*T*	—	*TD* and an unidentified spot
Cassowary (Table 1)	See Discussion	*T*	—	Entirely anomalous; see Discussion
King penguin and eagle-owl (Table 1)	*M* and *P*, see Discussion	*T*	—	*TC* and *TD*
Diamond rattlesnake, *Crotalus adamanteus* (20·6)	*M*. Almost certainly taurocholate	*T*	—	*TC*, possibly *TD*. Two or three unidentified spots
Python reticulatus (15·0)	*M*. Pythocholic and cholic acids	*T*	—	Spot slightly in advance of *TC*. ? Unidentified spot
Carpet python, *P. spilotus* (20·0)	*M*. Probably pythocholic acid	*T*	—	Spot in advance of *TC*. Probably *TD*
Australian crocodile, *Crocodylus johnsonii* (19·3)	Probably *P*	*T*	4	As cayman (see below)
Cayman, *Caiman crocodilus* (16·2)	*P*. Contains taurotrihydroxycoprostanate (Haslewood, 1952*a* and unpublished results)	*T*	4	Spots running faster than *TC* and at about the same rate as *TD*
Green turtle, *Chelone midas* (20·2)	Possibly *P*. Contains unidentified bile acids (Haslewood & Wootton, 1950)	*T*	4	Two spots, running close to but more closely than *TC* and *TD*
Frog, *Rana temporaria* (19·5)	*MP*. Mainly ranol sulphate (Haslewood, 1952*b*)	*T*	5	Almost as *TC*. ? *TD*
Gaboon viper, *Bitis gabonica* (20·4)	Unidentified bile salts (Haslewood & Wootton, 1950)	*T*	5	Two spots running more slowly than but close to *TC* and *TD*
Dogfish (Table 1)	*MP*. See Table 1	*T*	5	*TC*. ? *TD*
Mirror carp, *cyprinus carpio* (20·8)	*MP*. (Haslewood, 1951)	*T*	6	Spots differ from *TC* or *TD*
Tench, *Tinca vulgaris* (19·1)	Unidentified; the tench is a close relative of the carp	*T*	6	As carp (above)
Cod, flounder, plaice, bass (see Table 1)	All certainly or probably *M*	*T*	6	*TC* and *TD*

* Spots corresponding to taurocholate or taurodeoxycholate cannot be taken as necessarily indicating the presence of these substances (see text).

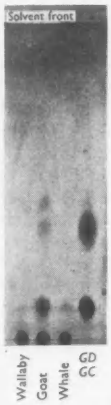

Fig. 1.

Fig. 2.

Fig. 3.

Figs. 1 and 2. Paper chromatograms of bile salts of species named. Ascending chromatography as described in Methods. Fig. 1: glyco system G_1. Fig. 2: glyco system G_2. *GD*, glycodeoxycholate standard (upper spot); *GC*, glycocholate standard (lower spot). Taurine conjugates remain at starting line. For interpretations, see Results and Table 2.

Fig. 3. Paper chromatograms of bile salts of pig and coypu. Descending chromatography as described in Methods, with use of glyco system G_3. Standards (from bottom of photograph upwards): *C*, cholic acid; *GD*, glycodeoxycholate; *GCh*, glycochenodeoxycholate; *GC*, glycocholate. For interpretations, see Table 2 and Discussion.

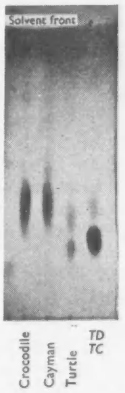

Fig. 4.

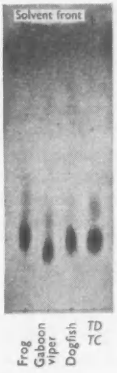

Fig. 5.

Fig. 6.

Figs. 4–6. Paper chromatograms of bile salts of species named. Descending chromatography as described in Methods, with use of tauro system *T*. *TD*, taurodeoxycholate standard (upper spot on photographs); *TC*, taurocholate standard (lower spot on photographs). For interpretations, see Table 2.

(i) C_{27} or C_{28} alcohols conjugated with sulphate ('most primitive'), (ii) C_{27} or C_{28} acids conjugated with taurine ('primitive'), or (iii) C_{24} acids conjugated with taurine or glycine ('modern').

Results are summarized in Table 2 and photographs of chromatograms are shown in Figs. 3–6.

DISCUSSION

Our failure to find glycine conjugates in the birds and fish examined tends to strengthen the working hypothesis of Haslewood & Wootton (1950) that 'glyco acids are apparently found only in mammalia'. However, it should be pointed out that in mammals bile salts containing a very high proportion of such conjugates seem to be found only in vegetarian species. For the present study, none of the few vegetarian reptiles and no herbivorous fish containing known bile salts were available; examination of such animals for glycine conjugates ought to be undertaken. Arnstein & Neuberger (1953) have suggested that in birds glycine is a 'semi-essential' amino acid, and amounts sufficient for the synthesis of glyco bile salts might not be expected to be readily available.

The results summarized in Table 2 call for certain detailed comments.

(*a*) The pig and coypu chromatograms (Fig. 3) each show spots corresponding to glycochenodeoxycholic acid, but not to glycodeoxycholic acid. Chenodeoxycholic acid has in fact been isolated from the bile of both animals (Ido & Sakurai, 1939; Haslewood, 1954). In the coypu, a spot corresponds to glycocholic acid; cholic acid has been found in the bile (Haslewood, 1954). In the pig, the 'glycocholic' spot ran at a somewhat slower rate than authentic glycocholic acid, suggesting that it might be due to a different substance; the phenomenon is certainly worth further investigation. The remaining spot in the pig chromatogram may be due to glycohyodeoxycholic acid and that in the coypu to glycoursodeoxycholic acid (Kazuno, 1946; Haslewood, 1954).

(*b*) It is evident from Fig. 3 and from unpublished chromatograms that pig and coypu bile salts contain small amounts of substances resembling taurodeoxycholate; tauro conjugation is probably not entirely suppressed in these species.

(*c*) The bile salts of the birds mentioned in this report are at present being examined by Dr I. G. Anderson in these laboratories, and the assessment of king penguin bile as showing both 'modern' and

'primitive' characteristics is due to him. Yamasaki (1951) has isolated a bile acid (probably C_{27}) from chicken bile salts, the chief constituent of which is, of course, taurochenodeoxycholate. Dr Anderson has found that our specimen of the 'bile salts' of the cassowary is indeed anomalous in its behaviour; he has suggested that it may consist largely of the fat and other materials normally excreted in variable amounts in bile.

(*d*) It is interesting that ranol sulphate (frog bile, Fig. 5) and the sulphate of scymnol (or a precursor) (dogfish, Fig. 5) both run at almost the same rate as taurocholate; the alcohol sulphate from the carp (Fig. 6) is not very different.

The simple methods of paper chromatography now tested should be of great value for the preliminary examination of bile salts; they have already indicated several lines along which progress may be made.

SUMMARY

1. Simple methods of paper chromatography developed by Sjövall (1952, 1954) have been applied to a search for bile acids conjugated with glycine in the bile salts of seven species of fish and eight species of birds and to the examination of the crude bile salts of a number of vertebrates.

2. No glycine conjugates were found in the fish and birds examined.

3. Some implications of the results have been discussed.

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The Phosphatides of the Latex of *Hevea brasiliensis*

2. PURIFICATION AND ANALYSIS

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Some properties of latex phosphatide were reported in a previous paper (Smith, 1954), but it was found that an extensive analysis could not be usefully carried out without first removing water-soluble impurities which were carried into solution in lipid solvents by the phosphatides. Workers with a variety of phosphatides from both animals and plants have encountered similar difficulties and have used a number of methods for purification. Thus Channon & Foster (1934), suspended wheat-germ phosphatide in water, and flocculated it by adding acetone and sodium chloride. This method was used by Tristram (1942) for the purification of latex phosphatide. Folch & Van Slyke (1939) and Christensen (1939) washed solutions of blood phosphatides in light petroleum with water. McKibbin & Taylor (1949) found that the large losses of phosphatide incurred by using this method could

be much reduced by using a solution of magnesium chloride for the washing. Folch (1949) and Sinclair (1948) dialysed emulsions of phosphatide, from brain and blood respectively, against distilled water. Water-soluble nitrogenous compounds (urea and choline were identified in certain cases) were usually the main contaminants detected, together with a small amount of water-soluble phosphorus. Folch (1949), however, found gross contamination of brain phosphatide with inorganic phosphates. Some of these methods have been examined with a view to using them for the purification of latex lipid but none was entirely satisfactory. The best results were obtained by using a partition-chromatographic technique which will be described.

Coagulation of latex in ethanol at a temperature below 40° has been previously shown (Smith, 1954) to lead to the enzymic liberation of choline from the phosphatide with an accompanying increase in its acetone solubility. Such a technique followed by acetone precipitation of the extracted lipid was used by most previous workers (Rhodes & Bishop, 1930; Tristram, 1942; Altman, 1946; Resing, 1951) for the preparation of phosphatide from latex. Thus the product examined by these workers was almost certainly an artifact from which, in addition, a fraction may have been lost by solution in acetone. These considerations do not necessarily apply to the method of preparation used by Altman & Kraay (1940) but their product was obtained, in very poor yield, from the silt left after centrifugal clarification of ammoniated latex, and was probably therefore not a representative sample of unchanged phosphatide. Tristram (1942), alone among the above workers, attempted to remove water-soluble impurities from the phosphatide, and this fact must also be borne in mind in assessing the value of analytical results obtained. In particular, the absence of such purification makes figures for total nitrogen valueless.

In the present work, samples of latex lipid, undegraded and degraded by the choline-liberating enzyme, have been prepared and analysed in a variety of ways before and after the removal of water-soluble impurities.

EXPERIMENTAL

Preparation of lipid samples. Seedling latex from the Experiment Station of the Rubber Research Institute of Malaya was extracted essentially according to the scheme outlined previously (Smith, 1954) but on a larger scale. In general, 1 l. batches of latex were coagulated in 3 l. of industrial redistilled methylated spirit (to be referred to as 'alcohol'). For the preparation of lipid in which the phosphatide was undegraded by the choline-liberating enzyme (to be referred to as undegraded lipid), the latex was added to boiling alcohol and the hot serum immediately filtered. When lipid containing phosphatide from which choline had been liberated enzymically (to be referred to as 'degraded lipid') was required, the latex was added to alcohol at 22° and the mixture stored at this temperature for 24 hr. before boiling for 15 min. and filtering off the hot serum.

Removal of water-soluble impurities from the lipid. Methods of purification involving the addition of metallic ions were rejected as being likely to lead to the formation of artificial metal phosphatidates. Water washing of a light petroleum (b.p. 56–70°) solution was unsatisfactory for the purification of undegraded lipid owing to the formation of persistent emulsions which resulted in losses of lipid of the order of 15% despite repeated re-extraction of the water washings with light petroleum. Only a small loss (about 3%) occurred with degraded lipid.

Purification by dialysis was carried out as follows: an aqueous suspension containing approximately 1% (w/v) of the lipid was placed in a cellophan sac and dialysed against distilled water. Nitrogen was slowly bubbled through the suspension and the dialysis was continued for 7 days at 10°, the water being changed twice daily. Analysis of the dialysable materials showed that there was no appreciable removal of nitrogen-containing compounds after this time. The purified emulsion was freeze-dried. This method was tedious to carry out and, as will be seen later, was inefficient. The following chromatographic technique was more satisfactory on both counts and was mostly used in these investigations.

Bevan, Gregory, Malkin & Poole (1951) reported that choline-containing phosphatides could be separated from ethanolamine- and serine-containing phosphatides by partition chromatography on cellulose using as a solvent the water-poor phase from a mixture of chloroform, ethanol and water (80:20:2·5, v/v), referred to as C.E.W. We found that paper chromatography of latex lipid with this solvent gave results similar to those of Bevan *et al.* (1951) in that a ninhydrin-reacting spot near the origin (R_F value about 0·01) and a choline-containing lipid spot at or near the solvent front were obtained. Further examination showed, however, that the former spot was not due to a phosphatide, but to a mixture of water-soluble substances, mainly known amino acids. It appeared therefore that this technique might form the basis of a method of removing water-soluble impurities. Fuller examination, the results of which will be given in a later section, confirmed this view.

Two methods of treatment were used. (*a*) The lipid (about 30% (w/v) solution in $CHCl_3$) was spotted 8 cm. from the top of a 30 cm.-long strip of filter paper (Whatman no. 1). This was developed with C.E.W. at 22°, using the ascending technique in an airtight gas jar, until the solvent front had travelled about 15 cm. beyond the starting line. The main lipid band, near the solvent front, was visible to the naked eye in transmitted light after drying at room temperature. A transverse strip containing this material was cut out and extracted with $CHCl_3$. The remaining part of the chromatogram was extracted first with water and then with $CHCl_3$. The $CHCl_3$ extractions were carried out in a Soxhlet extractor after cutting the paper into small pieces. The water extraction was made by cutting one end of the strip to a point, then immersing the other end in a trough of water contained in a simple descending type paper chromatographic cabinet (Dent, 1948) and collecting the drippings. (*b*) Whatman cellulose powder (standard grade, ashless) was slurried with enough C.E.W. solvent to cover it. This, and all subsequent operations, were carried out in a constant-

temperature room at 22°. A wad of glass wool was placed in the bottom of a suitably sized tube fitted with a tap and the slurry added. After the cellulose powder had settled under gravity, the solvent was run out until its level was just above that of the cellulose powder. The liquid level was never allowed to fall below this point. The lipid, dissolved in C.E.W. solvent, was added and the tap opened to allow a dropwise flow (about 1–10 ml./min. depending on the column size). The chromatogram was developed with C.E.W. solvent until all the phosphatide had emerged from the column. Successive washings with $CHCl_3$, ethanol and water in that order were then carried out, each fraction being collected separately in an attempt to recover materials held on the column. It was found that, provided the ratio of lipid weight:column volume was less than 1:25, a further passage of the purified lipid through a cellulose-powder column caused no additional change in its composition. Although the C.E.W. solvent of Bevan *et al.* (1951) was used in all the experiments reported, it was subsequently found that $CHCl_3$ saturated with water could be used equally well.

Total phosphorus, ash phosphorus, choline and total nitrogen determinations. The first three were carried out as described previously (Smith, 1954). Total nitrogen was determined by the micro-Kjeldahl method using a copper—selenium catalyst for the digestion.

Reducing-sugar determination. This was carried out colorimetrically according to Somogyi (1945), the results being calculated as galactose. A lipid sample (about 0·1 g.) was hydrolysed by refluxing for 24 hr. with 20 ml. of 2N-H_2SO_4. The mixture was cooled and filtered and the filtrate was neutralized with 4N-NaOH. The resultant concentration of Na_2SO_4 was shown to have no influence on the determination. Some sugar destruction probably occurred but higher results were obtained with this procedure than with the milder H_2SO_4 hydrolysis of Scholfield, Dutton & Dimler (1952).

Determination of unsaponifiable matter and total fatty acid. The lipid (about 0·03–0·05 g.) was hydrolysed, and the unsaponifiable matter and fatty acids were extracted in the manner described by Bloor (1943). This extract was evaporated and then dried under vacuum to constant weight. The residue was taken up in neutral ethanol, heated to boiling, and titrated with 0·02N-NaOH using phenolphthalein as indicator. To calculate the respective amounts of fatty acids and unsaponifiable matter in the mixture, we assumed a mean molecular weight of 325 for the fatty acids. This value, which was based on the results of Tristram (1942), may not be correct. It is higher than might be expected, and although it is assumed that Tristram (1942) separated unsaponifiable matter from his fatty acids before determining their molecular weight, he makes no mention of doing so.

meso*Inositol.* This was determined microbiologically according to Smith (1951). A lipid sample was hydrolysed by refluxing with 5N-HCl for 48 hr. The cooled mixture was filtered and the filtrate evaporated to dryness. The residue, after removal of most of the HCl under vacuum, was taken up in water and neutralized with NaOH. Inositol was shown to be stable under these conditions, but complete liberation from a combined form might not have occurred. However, higher values were obtained by this method than by 48 hr. hydrolysis with either 2N-H_2SO_4 or saturated methanolic $Ba(OH)_2$.

Chromatographic examination of ninhydrin-reacting substances. Lipid hydrolysates were prepared in the same way as those for inositol determination except that, in order to remove traces of HCl, the residues were repeatedly taken up with water, re-evaporated and kept under high vacuum in the presence of KOH. The final residues taken up with water contained about 75% of the non-choline nitrogen in the lipid. Hydrolysis for 48 hr. with 2N-H_2SO_4 and saturated methanolic $Ba(OH)_2$ gave recoveries of non-choline nitrogen of only 44 and 23%, respectively. The hydrolysates, with and without the addition of known amino acids as reference substances, were submitted to two-dimensional chromatography on Whatman no. 1 filter paper (44·5 × 57 cm.) at a constant temperature (30°), essentially according to Dent (1948).

RESULTS

Purification of latex lipid

Paper chromatographic separation of unpurified undegraded lipid gave the fractions shown in Table 1. Lipid, representing about 94% of the total was recovered from the strip near the solvent front. It contained a smaller percentage of nitrogen than the original lipid but nearly all of the nitrogen lost was recovered in a water-soluble form from the upper part of the paper, and only a trace was soluble in chloroform.

Partition chromatography on a cellulose column permitted separation on a sufficiently large scale for detailed analysis of the fractions (Table 2). Samples of undegraded and degraded unpurified lipid, respectively, were treated, and in both cases it was

Table 1. *Properties of undegraded, impure latex lipid and of fractions obtained therefrom by paper chromatographic separation using* C.E.W. *solvent*

Four papers (57 × 30 cm.) were used, each bearing 40 × 0·01 ml. spots of an approximately 30% (w/v) light petroleum solution of the lipid. The spots were added at 1 cm. intervals along a line 8 cm. from a longer edge of the paper. Strip *B* contained only the main lipid band near the solvent front which was sharply visible in transmitted light. Strip *A* consisted of the rest of the chromatogram.

		Lipid (g.)	Total P (mg.)	Total N (mg.)	Choline N (mg.)	Lipid N/P atomic ratio	Lipid choline N/P atomic ratio
	Unpurified lipid	0·485	9·23	5·34	2·71	1·28	0·65
Strip *A*	Water-soluble	—	0·51	2·01	0	—	—
	$CHCl_3$-soluble	—	0·40	<0·05	0	<0·3	0
Strip *B*	$CHCl_3$-soluble	0·454	8·13	3·40	2·53	0·93	0·69

found that lipid, representing about 95% of the total by weight, passed through the column with the solvent (in all, 17·48 and 14·33 g., respectively, of undegraded and degraded lipid were treated and 16·46 and 13·77 g., respectively, passed through the column). After each chromatogram had been developed as described in Table 2, it was washed with 400 ml. chloroform, 750 ml. absolute ethanol and 2000 ml. water in that order, the effluents being collected separately. The chloroform effluent contained only about 2% of the total material retained on the column. The ethanol effluent was evaporated down, after the addition of water, to give an aqueous residue which was added to the water effluent. The recovered materials, which were entirely water-soluble, accounted approximately for the loss in weight on passing the lipid through the column (in all, 1·14 and 0·61 g. water-soluble materials were recovered after treating the undegraded and degraded lipid, respectively). They contained 1·5 and 1·1% phosphorus, 3·1 and 3·4% nitrogen (but no choline), 1·6 and 1·6% inositol, 3·3 and 4·9% reducing sugar and 49 and 64% ash (as phosphate) for the undegraded and degraded lipids, respectively.

The metallic impurities, responsible for the ash content of the water solubles, were not soaps, since no free fatty acids could be detected after acidification. Lipid samples from a variety of latices contained impurities in amounts similar to those shown here with the exception that water-soluble nitrogen was found to vary from about 0·15 to 0·5% depending on the lipid. The nitrogenous impurity never included choline.

The ashes from the purified undegraded and degraded lipids, which were prepared by heating in a muffle at 500° for about 24 hr., contained respectively 22·1 and 23·8% potassium as K_2O; 2·7 and 6·0% magnesium as MgO and 44·2 and 49·0% phosphorus as P_2O_5. The remaining constituents were not identified. Sodium and calcium were present in not more than trace amounts but there was an unknown substance which gave a small dark-coloured precipitate with cobaltinitrite during the potassium determination.

When the purified lipids were hydrolysed by refluxing with 0·1 N alcoholic NaOH for 1 hr. about 97% of the phosphorus was recovered in a water-soluble organic form. None was liberated in an inorganic form on heating a 5 ml. sample of this solution, containing about 0·03 mg. phosphorus, with 1 ml. 10 N-H_2SO_4 in a boiling water bath for 1 hr. Similar treatment with the addition of 1 ml. 0·05 M periodic acid (Burmaster, 1946) gave recovery of 89–91% of the phosphorus in an inorganic form for both the undegraded and degraded lipins. Glycerophosphate is broken down quantitatively under these conditions so that part of the lipid

Table 2. *Effect of purification by dialysis and cellulose column partition chromatography respectively on the properties of undegraded and degraded latex lipid*

For the first method approximately 100 ml. batches of 1% (w/v) aqueous suspensions of the lipids were dialysed against distilled water. For the second method 7–8 g. batches of the lipids, dissolved in 50 ml. C.E.W. were added to cellulose-powder columns (4 cm. diam. × 25 cm. length) and washed through by a further 750 ml. C.E.W. The last 200 ml. of effluent contained negligible amounts of phosphorus (about 0·25% of the total). For further details of both procedures see text.

	Total P (%)	Total N (%)	Ash P (%)	Choline N (%)	Non-choline N (%)	N/P (atomic ratio)	Choline N/P (atomic ratio)	Ash-P/ total-P (ratio)	N/P + ash-P/ total-P	Total ash (%)	Inositol (%)	Reducing sugar (%)	Total fatty acid (%)	Unsaponifiables (%)
Undegraded lipid														
Unpurified	1·90	0·91	0·78	0·57	0·34	1·06	0·67	0·41	1·47	4·14	1·34	8·94	53	16
Purified (dialysis)	1·94	0·81	0·62	0·59	0·22	0·93	0·68	0·27	1·20	2·93	—	—	—	—
Purified (chromatography)	1·82	0·72	0·33	0·59	0·13	0·88	0·72	0·18	1·06	1·71	1·30	8·60	55	14
Degraded lipid														
Unpurified	1·91	0·42	1·67	0·07	0·35	0·49	0·08	0·88	1·37	7·91	0·80	8·14	54	18
Purified (dialysis)	1·95	0·28	1·20	0·06	0·22	0·32	0·07	0·62	0·94	6·05	—	—	—	—
Purified (chromatography)	1·79	0·21	1·32	0·06	0·15	0·26	0·08	0·74	1·00	6·15	0·72	7·84	56	15

phosphorus does not appear to be present as glycerophosphate. It is not clear in what form it occurs. It would be expected that inositol monophosphate, if present, would react in the same way as glycerophosphate.

Analytical results for lipid samples after dialysis in aqueous suspension against distilled water are also given in Table 2. In all, 15·98 and 15·28 g., respectively, of the undegraded and degraded lipid were treated and 15·39 and 14·13 g., respectively, were recovered. Part, at least, of the lipid loss was mechanical. The procedure did not remove any appreciable amount of water-soluble phosphorus and the amounts of nitrogen removed were less than by chromatographic purification, indicating a less efficient purification by dialysis. The ash content of the undegraded lipid also indicated that the chromatographic purification was the more efficient but the ash content of the degraded lipid was reduced to a greater extent by dialysis. It may be that certain of the phosphatidic acid salts, present in the latter lipid as degradation products of the choline containing phosphatide, are somewhat soluble in water (Witcoff, 1951). If this were so the setting up of successive Donnan equilibria for each change of water could result in the loss of metallic ions therefrom and the consequent artificial formation of free phosphatidic acid.

It has been shown (Smith, 1954) that the coagulation in alcohol of amounts of latex greater than about 40 ml. may lead to inefficient lipid extraction owing to the lipid being trapped within the bulky rubber coagulum. This is particularly apparent in the preparation of degraded lipid when the latex is coagulated in the cold and the mixture only subsequently heated to boiling and filtered. In the large-scale extractions used for the preparation of lipids, for which analysis figures are given in Table 2, the calculated yields on the latex (allowing for serum not recovered from the coagulum) were 0·73 and 0·60 % for the undegraded and degraded unpurified lipids, respectively. The difference may well be due to less complete removal of phosphatide, insoluble in cold ethanol, in the latter case. We believe that this explains the differences in inositol and reducing-sugar contents between the undegraded and degraded lipids. This belief was supported by a further experiment in which undegraded and degraded lipid samples were prepared from 40 ml. lots of latex in the manner described previously (Smith, 1954). In this way, calculated lipid yields of 0·71 and 0·73 % were obtained for the undegraded and degraded unpurified lipids, respectively. These lipids contained 1·89 and 1·91 % phosphorus, 0·55 and 0·10 % choline nitrogen, 1·31 and 1·34 % inositol and 9·8 and 10·1 % reducing sugar for the former and latter, respectively.

Fractionation of the lipids according to acetone and ethanol solubility

Latex phosphatide, particularly when degraded by the choline-liberating enzyme, has been shown to be soluble in acetone (Smith, 1954). Consequently although it was impossible to obtain the total phosphatides free of non-phosphatide lipid by this means, we considered that useful data concerning the components of the phosphatide mixtures might be obtained by fractionating with acetone. Further information was sought by fractionating the acetone insolubles with ethanol. Complete separation of phosphatide mixtures by making use of their solubility differences is very difficult even using a counter-current extraction technique (e.g. Lovern, 1952). Consequently it was thought that more useful information would be obtained in the first instance by carrying out a simple separation so that quantitative recovery of the lipid could be easily effected, rather than by attempting to isolate pure components. Table 3 shows the properties of fractions from the undegraded and degraded latex lipid purified by partition chromatography and described in Table 2. The fractions were prepared by adding 6 vol. acetone to 30 ml. of a 15 % (w/v) solution of the lipid in light petroleum (b.p. 56–70°), keeping at 10° overnight and pouring off the supernatant liquid after centrifuging. Absolute ethanol (20 vol.) was rapidly added to the washed residue (the dry residue discoloured and became partially insoluble in light petroleum after only a few minutes exposure to the air). The mixture was heated to boiling under nitrogen with constant shaking, cooled to 10° and kept overnight at this temperature. The supernatant liquid was decanted off after centrifuging, and the washed residue was dissolved in light petroleum. As was to be expected, there was no clear-cut separation of the different components of the mixture.

A small amount of undegraded phosphatide and a larger amount of degraded phosphatide were present, together with the bulk of the non-phosphatide lipids, in acetone solution. In another experiment, 0·3 g. samples of the total lipids dissolved in 1 ml. light petroleum were precipitated with 6 ml. acetone and 3 drops of a saturated ethanolic solution of magnesium chloride (Bloor, 1929). The solutions were evaporated to 1 ml. and the precipitation repeated. Under these conditions 18·3 % of the undegraded lipid was soluble in acetone and this fraction contained only 0·012 % phosphorus ($<0·2$ % of the total lipid phosphorus) but 0·125 % nitrogen. Almost all of the latter must therefore be non-phosphatide. The degraded lipid gave 37·7 % acetone-solubles containing 0·23 % phosphorus and 0·089 % nitrogen. Although all the phosphatide was not precipitated in this instance it

Table 3. *Properties of fractions obtained from chromatographically purified lipids according to their acetone and ethanol solubility*

The properties of the total lipids are given in Table 2. Fractions were obtained by single precipitations and quantitative recovery of the lipids. For details see text.

	Percent of total lipid	Total P (%)	Total N (%)	Ash P (%)	Choline N (%)	Non-choline N (%)	N/P (atomic ratio)	Choline N/P (atomic ratio)	Ash-P/ total-P (ratio)	N/P + ash-P/ total-P	Inositol (%)	Reducing sugar (%)	Total fatty acid (%)	Unsaponifiables (%)
Undegraded lipid														
Acetone-soluble	25·2	0·40	0·27	0·03	0·17	0·10	1·50	0·92	0·07	1·57	0·07	4·4	34	51
Acetone-insoluble: Ethanol-soluble	62·0	2·20	0·91	0·15	0·88	0·03	0·92	0·89	0·07	0·99	0·54	12·2	64	1
Acetone-insoluble: Ethanol-insoluble	12·8	2·48	0·43	1·62	0·22	0·21	0·38	0·20	0·65	1·03	8·17	11·2	56	10
Degraded lipid														
Acetone-soluble	45·0	0·81	0·17	0·29	0·04	0·13	0·48	0·11	0·36	0·84	0·04	4·2	51	32
Acetone-insoluble: Ethanol-soluble	39·5	2·45	0·20	1·87	0·12	0·08	0·18	0·11	0·76	0·94	0·13	12·9	65	0
Acetone-insoluble: Ethanol-insoluble	15·5	3·10	0·19	2·65	0	0·19	0·14	0	0·86	1·00	4·06	12·0	67	8

can be justifiably assumed that virtually all nitrogenous phosphatide was removed so that the nitrogen in this fraction must also be non-phosphatide. Titration of an ethanolic solution of the acetone-soluble material from the undegraded phosphatide, after water washing to remove traces of magnesium chloride, showed no free fatty acids to be present. This determination was not possible with the degraded lipid owing to the presence of phosphatides in the acetone-solubles.

Chromatographic examination of ninhydrin-reacting substances

Fig. 1*a* shows that the impure undegraded lipid, for which analysis figures are given in Table 2, contains a number of ninhydrin-reacting substances including some known amino acids and ethanolamine. Among the former, only serine has been previously reported as a phosphatide constituent (e.g. Hutt, Malkin, Poole & Watt, 1950). Closely similar chromatograms were obtained with the hydrolysates of a number of unpurified latex lipid samples examined. Although dialysis failed to remove most of the ninhydrin-reacting materials from the lipids (Fig. 1*b*) purification on the cellulose column removed all but traces of all of them except ethanolamine (Fig. 1*c*). These substances, again with the sole exception of ethanolamine, were detected in a free form in the water-soluble impurities recovered from the column (Fig. 1*d*). Results obtained using the degraded lipid were apparently identical with those for the undegraded material and the chromatograms obtained are consequently not illustrated. It is possible that there were undetected quantitative differences. Examination of the fractions obtained by the paper chromatographic purification of undegraded lipid (Table 1) gave similar results.

Hydrolysates of the acetone/ethanol lipid fractions, properties of which are given in Table 3, all gave a strongly marked ethanolamine spot together with trace spots of all or some of the amino acids. Qualitative results were identical for the undegraded and degraded lipids.

DISCUSSION

The statement of Bevan *et al.* (1951) that a separation of choline-containing phosphatides from other nitrogen-containing phosphatides could be achieved by partition chromatography on cellulose using a chloroform/ethanol/water solvent has not been confirmed. Thus although a considerable amount of nitrogenous material, including certain known amino acids and other ninhydrin-reacting substances, was retained by the stationary phase on treating latex lipid in this way (using either paper or a cellulose-powder column) this nitrogenous

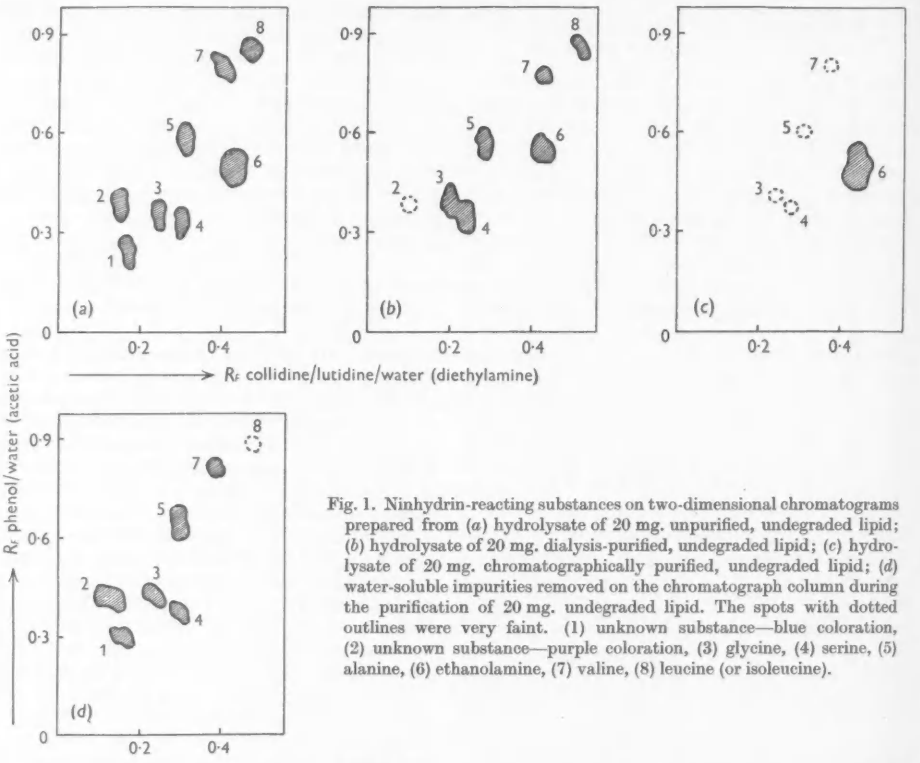

Fig. 1. Ninhydrin-reacting substances on two-dimensional chromatograms prepared from (*a*) hydrolysate of 20 mg. unpurified, undegraded lipid; (*b*) hydrolysate of 20 mg. dialysis-purified, undegraded lipid; (*c*) hydrolysate of 20 mg. chromatographically purified, undegraded lipid; (*d*) water-soluble impurities removed on the chromatograph column during the purification of 20 mg. undegraded lipid. The spots with dotted outlines were very faint. (1) unknown substance—blue coloration, (2) unknown substance—purple coloration, (3) glycine, (4) serine, (5) alanine, (6) ethanolamine, (7) valine, (8) leucine (or isoleucine).

material was almost completely recoverable in a water-soluble form together with a number of other water-soluble substances. The lipid not retained on the paper or column contained, as well as choline, ethanolamine in roughly the same amount as in the untreated material. The technique appears to provide an efficient method of removing water-soluble impurities of a varied nature from the lipid. Unless egg phosphatides behave differently from latex phosphatides, it seems that Bevan *et al.* (1951) may have mistaken ninhydrin-reacting impurities for ninhydrin-reacting phosphatides. Since completing this work, a paper by Lea & Rhodes (1953) has come to our notice. These workers repeated the experiments of Bevan *et al.* (1951) and found evidence that the slowly moving ninhydrin-reacting substances in egg-yolk phosphatides were due to water-soluble impurities including known amino acids. They also found an unknown substance giving a blue coloration with ninhydrin (cf. Fig. 1*a* and *d*).

An examination of lipid samples, purified by this method, has confirmed the observation of Tristram (1942) that much of the reducing sugar associated with the phosphatide is not easily removed in a water-soluble form. Inositol, detected by Resing (1951) in latex phosphatide from which water-soluble impurities were not removed, has been found in the purified material; but serine, also reported to be present by Resing (1951), appears to be a water-soluble impurity.

The undegraded and degraded purified lipids show marked differences apart from the loss of choline. Variations in inositol and reducing sugar appear, however, to be due to incomplete extraction from the latex rather than to intrinsic differences in the lipid. The only other obvious change on degradation is an increase in ash content. This was indicated previously (Smith, 1954), but the presence of impurities rendered the results of doubtful value. Further information can be derived from a study of the nitrogen/phosphorus and ash phosphorus/total phosphorus ratios of the purified lipids and their acetone/alcohol fractions. The phosphatides likely to be present can be put into one of three classes, irrespective of the main structure of the molecule, according to the substituent groups combined with the phosphate radical. These are (i) nitrogenous

phosphatides containing equimolecular amounts of nitrogen and phosphorus, (ii) metal phosphatidates and (iii) free phosphatidic acids. Ashing of a phosphatide mixture, from which complete removal of contaminating metal salts had been achieved, would result in the complete loss, by volatilization, of the phosphorus in any wholly organic phosphatide. The phosphorus in any metal phosphatidate present, on the other hand, would be quantitatively retained in the ash. Thus the ash phosphorus/total phosphorus ratio of the mixture would give the proportion of phosphatide phosphorus present as metal phosphatidate. Similarly, in a phosphatide mixture free of nitrogenous impurities, the nitrogen/phosphorus atomic ratio would give the proportion of phosphatide phosphorus present as nitrogenous phosphatide. The sum of these two ratios, which will be subsequently referred to as the (N/P + ash-P/P) sum, should be unity if free phosphatidic acids are absent and less than unity if they are present. The extent to which this sum is less than unity would give the proportion of phosphatide phosphorus present as free phosphatidic acids. The total undegraded and degraded purified lipids are not completely free of non-phosphatide nitrogen. They contain traces of amino acids, although the amounts must be very small and are probably negligible; more important they contain acetone-soluble non-phosphatide nitrogen. The undegraded and degraded total lipids possess (N/P + ash-P/P) sums of 1·03 and 0·96, respectively, after correction for this material. This indicates the possible presence of free phosphatidic acid in the degraded lipid although the effect is small enough to be accountable as experimental error. The finding is confirmed, however, by the fact that the acetone-soluble fraction from the degraded lipid possesses a (N/P + ash-P/P) sum of considerably less than unity even before correction for known non-phosphatide nitrogen. When this correction is made, a sum of only 0·61 is obtained, indicating the presence of 39% phosphatide phosphorus as free phosphatidic acid. It is presumably this substance which is largely responsible for the high acetone solubility of the degraded phosphatide. The corresponding fraction from the undegraded lipid has a (N/P + ash-P/P) sum of 1·09 after correcting for known non-phosphatide nitrogen. All the acetone-insoluble fractions obtained, with the exception of the ethanol-soluble part from the degraded lipid which appears to contain a small amount of free phosphatidic acid, possess (N/P + ash-P/P) sums close to unity. It seems probable therefore that the phosphatides in these fractions are made up almost entirely of nitrogenous phosphatides and metal phosphatidates and that no appreciable nitrogenous or metallic impurities are present. Traces of amino acid impurities were detected in all the fractions but the amounts are apparently negligible.

It appears probable from the manner in which the various phosphatide constituents distributed themselves in the acetone/ethanol fractions that the phosphatides in the undegraded lipid consist of (i) an essentially ethanol-soluble phosphatide containing choline (presumably lecithin) and associated with reducing sugar, (ii) an essentially ethanol-insoluble metal phosphatidate containing inositol also associated with reducing sugar, and (iii) a non-choline nitrogenous phosphatide which from the chromatographic results appears to be a phosphatidyl ethanolamine. It is possible that the ethanolamine occurs in combination with the inositol phosphatide as, according to Woolley (1943), is the case in 'lipositol' from soya beans. The non-choline nitrogen figures are quite unreliable as a means of estimating the amount of ethanolamine—they are measured as small differences between two large values and residual amounts of nitrogenous impurity are almost certainly present, which although small in relation to the total lipid may be large in comparison with the small amount of ethanolamine. However, since chromatographic examination showed strong ethanolamine spots in fractions with very little inositol, the suggestion of a separate phosphatidyl ethanolamine is preferred. The phosphatides in the degraded lipid appear also to contain these three components although with the first in much reduced amount. It has previously been shown that some free phosphatidic acid is present in this lipid. In addition, the amount of ash phosphorus is much more than can reasonably be accounted for as an inositol-containing metal phosphatidate, particularly in the acetone-insoluble, ethanol-soluble fraction. It seems that a further metal phosphatidate, not containing inositol, must be present. These last two products are presumably formed from the lecithin as a result of the action of the choline-liberating enzyme. As well as the phosphatides, the lipids contain unsaponifiable material and, since in the acetone-soluble fractions there was far more fatty acid than could reasonably be attributed to the phosphatide they must also contain other lipids, e.g. triglycerides or free fatty acids. There were no free fatty acids in the undegraded lipid and probably not in the degraded lipid although they were not determined in the latter. It is possible that part of the extra fatty acid was present, not as triglyceride, but in combination with unsaponifiable matter; the nature of the unsaponifiable matter has not, however, been ascertained.

Our results show that latex lipid is a mixture containing a number of more or less ill-defined components. With the present data it is not possible to derive quantitative information concerning the composition of this mixture without making

numerous assumptions of varying degrees of reliability. It must be stressed, therefore, that the following deductions are tentative, although we consider it likely that, with the probable exception of the figures for phosphatidyl ethanolamine, they are substantially correct. The assumptions made are as follows: (i) All the non-choline nitrogen present (excepting the acetone-soluble non-phosphatide nitrogen) is due to ethanolamine which is present in a phosphatidyl ethanolamine of normal structure. As previously stated the ethanolamine may not occur in this form and even if it does it is unlikely that an accurate estimate is obtained on this basis. The amount is so small, however, that an error would not greatly affect the calculation of the remaining constituents. (ii) All the phosphatides present possess fatty acid/phosphorus molecular ratios of 2/1. This is theoretically true for lecithin and phosphatidyl ethanolamine and was also found by Woolley (1943) to be approximately true for the inositol-containing phosphatide from soya beans. The experimental results show that this ratio, even for the acetone insoluble fractions, is rather more than 2/1. The excess of fatty acid is regarded as being present as non-phosphatide lipid (e.g. triglyceride), precipitated from acetone solution with the phosphatides. (iii) The fatty acids have an average molecular weight of 325 (Tristram, 1942). (iv) The nitrogenous phosphatides possess a nitrogen/phosphorus ratio of 1/1. (v) The inositol-containing metal phosphatidate has an ash phosphorus/total phosphorus ratio of 1/1. The unsaponifiable contents of the fractions are known and the non-phosphatide fatty acid and phosphatidyl ethanolamine contents can be calculated by making the above assumptions. Calculation by difference shows that the remaining phosphatide mixtures in the acetone-insoluble fractions, both ethanol-soluble and ethanol-insoluble, contain about 2·8% phosphorus. This, despite the fact that in one fraction the majority is lecithin and in the other the majority is metal phosphatidate containing inositol. It appears therefore that both the lecithin and the inositol phosphatide contain nearly the same phosphorus percentage (about 2·8%) and their relative amounts in the fractions can consequently be calculated from the choline nitrogen/phosphorus and the ash phosphorus/total phosphorus ratios. By accepting these values the approximate inositol and reducing-sugar contents of the individual phosphatides can be deduced.

From the derived properties of the lecithin it is possible to calculate approximate properties for the free phosphatidic acid and its metallic salts (as potassium phosphatidate) which are apparently formed in the degraded lipid as a result of simple loss of choline from the lecithin. Accepting these properties and making the same assumptions as

Table 4. *Estimated composition of the lipids and their acetone/ethanol fractions*

The estimation is based on the results in Tables 2 and 3 making the assumptions described in the text (on this page). Values are expressed to the nearest 0·5%. The difference between the amounts of inositol phosphatide in the undegraded and degraded lipids is believed to be due to inefficient extraction of the latter from the latex rather than to any intrinsic difference in the lipids.

		Percentage (w/w) of total lipid						
	Total fraction	Lecithin	Phosphatidyl ethanolamine	Inositol phosphatide	Free phosphatidic acid*	Metal phosphatidate*	Tri-glyceride†	Unsaponifiables
Undegraded lipid								
Acetone-soluble	25	3·5	0·5	0	0	0	7·5	13·5
Acetone-insoluble { Ethanol-soluble	62	45·5	1·0	3·0	0	0	12·0	0·5
Acetone-insoluble { Ethanol-insoluble	13	2·0	1·5	7·5	0	0	0·5	1·5
Total	—	51·0	3·0	10·5	0	0	20·0	15·5
Degraded lipid								
Acetone-soluble	45	1·5	1·5	0	5·0	5·0	17·0	15·0
Acetone-insoluble { Ethanol-soluble	39·5	4·0	2·0	0·5	2·0	25·0	6·0	0
Acetone-insoluble { Ethanol-insoluble	15·5	0	1·5	4·5	0	8·0	0·5	1·0
Total	—	5·5	5·0	5·0	7·0	38·0	23·5	16·0

* Breakdown products from lecithin.

† Representing fatty acid in excess of that attributable to phosphatide.

before, a quantitative estimation of the components in the degraded lipid can be made.

Calculated values for the compositions of the undegraded and degraded lipids and their acetone/ethanol fractions are given in Table 4.

If these values are correct it can be calculated that the lecithin component contains reducing sugar (calculated as hexose) and phosphorus in a roughly equimolecular ratio. The inositol-containing metal phosphatide has been assumed to possess a fatty acid/phosphorus molecular ratio of 2/1 (an assumption which is supported by the fact that the experimentally determined fatty acid/phosphorus ratio of the ethanol-insoluble fraction of the undegraded lipid which appears to consist largely of this phosphatide is 2·16/1). Calculations indicate that it contains both inositol and reducing sugar in roughly equimolecular proportions to phosphorus. These findings are in close agreement with those for the soya bean 'lipositol' of Woolley (1943).

Comparison of our results, with the results of previous workers, gives further evidence that the phosphatides which they examined were partially degraded by the choline-liberating enzyme. Thus Tristram (1942) found about 35 % lecithin and 35 % phosphatidic acid (or its salts) in his acetone-insoluble lipid. Corresponding estimated figures for our undegraded and degraded products, respectively, were 63 and 7 % lecithin and 14 and 73 % phosphatidic acid (or its salts—including the inositol containing phosphatidate). Altman (1946), fractionating total lipid, found 45 % acetone-soluble, 33 % acetone-insoluble, ethanol-soluble and 22 % acetone-insoluble, ethanol-insoluble. These figures are close to our findings for degraded lipid.

SUMMARY

1. The statement of Bevan *et al.* (1951) that choline-containing phosphatides are separated from non-choline nitrogenous phosphatides by partition chromatography on cellulose could not be confirmed. Instead it was found that the removal of water-soluble impurities from latex lipid, which is usually difficult to achieve was efficiently accomplished by this technique.

2. Inositol, reducing sugar and substances containing nitrogen, phosphorus and metals, were shown to be present partly as water-soluble impurities in unpurified latex lipid preparations. The nitrogenous impurities contained certain known amino acids but did not contain ethanolamine or choline.

3. Evidence is presented which tentatively suggests that purified undegraded latex lipid consists of (i) lecithin containing reducing sugar (51 %); (ii) metal phosphatidate containing inositol and reducing sugar (10·5 %); (iii) phosphatidyl ethanolamine (3 %); (iv) triglyceride (representing fatty acid in excess of that attributable to phosphatide) (20 %); and (v) unsaponifiables (15·5 %).

4. The enzymic liberation of choline from the lecithin fraction leads to the formation of free phosphatidic acid and its metallic salts. No other marked changes are apparent.

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The Phosphatides of the Latex of *Hevea brasiliensis*

3. CARBOHYDRATE AND POLYHYDROXY CONSTITUENTS

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It has long been recognized that vegetable phosphatides usually contain carbohydrates (Winterstein & Hiestand, 1906). It seems frequently to be true that although part of this carbohydrate may be present as water-soluble impurity, some is more firmly bound with the phosphatide and can only be removed therefrom by hydrolysis. Recently, for example, Scholfield, Dutton & Dimler (1952) found that sucrose, stachyose and raffinose could be extracted from soya bean 'lecithin' with aqueous ethanol but that galactose, mannose and arabinose were not removed in this way and were apparently bound to the phosphatide. Latex phosphatide is no exception to the general rule and the initial finding of Rhodes & Bishop (1930), that it contained reducing sugar, has several times been confirmed. Tristram (1942) found that sugar was retained by the phosphatide even after the removal of water-soluble impurities and we confirmed this (Smith, 1954*b*) showing, however, that some free reducing sugar impurity was present. Resing (1951), examined by paper chromatography hydrolysates from latex phosphatide which had not been freed of water-soluble impurities; he identified galactose, glucose and a substance which he considered to be a polysaccharide. He also detected *meso*inositol by this means, a finding which we have confirmed by microbiological assay (Smith, 1954*b*). We also found that although most of the *meso*inositol was firmly bound to the lipid, a small amount was removable in a free water-soluble form.

Evidence has been obtained by Folch & Woolley (1942) and Woolley (1943), respectively, which suggests that *meso*inositol is present in a chemically combined form in brain and soya bean phosphatides. It is reasonable to assume a similar combination in other inositol-containing phosphatides but the manner in which carbohydrates are bound is more obscure. Phosphatides have a great capacity for forming complexes of uncertain type with various substances. The subject has been reviewed by Witcoff (1951). In some of these complexes, the binding is believed to be adsorptive rather than chemical (*e.g.* in the lecithin/cholesterol complex (Price, 1933)) and despite the close association between carbohydrate and the vegetable phosphatides, there is still some doubt as to whether this association is physical or chemical.

In the present work, the free and bound carbohydrate and polyhydroxy constituents of latex lipid have been examined by paper chromatography. The compositions of certain products obtained by partial hydrolysis of the lipid have given evidence that both *meso*inositol and certain carbohydrates are present in the phosphatides in a chemically combined form.

A summary of the findings reported in this paper and in the preceding papers of the series, with a discussion of possible practical implications in latex technology, has been published elsewhere (Smith, 1953).

EXPERIMENTAL

Lipid samples. Previous results (Smith, 1954*b*) have shown that virtually all the bound reducing sugar and *meso*inositol in latex lipid is associated with phosphatide. Consequently, most of the present experiments were carried out on total latex lipid, although fractions obtained by acetone and ethanol precipitation were sometimes used. Lipid samples, undegraded and degraded by the choline-liberating enzyme (Smith, 1954*a*) were examined both in a crude state and after purification by dialysis or partition chromatography. Sometimes, the water-soluble impurities removed were themselves examined. The details of lipid preparation, removal and recovery of water-soluble impurities and fractionation according to acetone and ethanol solubility have been described previously (Smith, 1954*b*).

Preparation of hydrolysates. Complete hydrolysis was carried out by refluxing about 0·5 g. of the lipid with 20 ml. 2 N-H_2SO_4 for 48 hr. The filtered solution was nearly neutralized with solid $Ba(OH)_2$. Neutralization was completed with 0·1 N-$Ba(OH)_2$ and the $BaSO_4$ filtered off. Partial hydrolysis was effected by refluxing about 0·5 g. lipid for 1 hr., either with 20 ml. of 0·01 N-H_2SO_4 or with 20 ml. of a saturated methanolic solution of $Ba(OH)_2$. The turbid filtrate from the former reaction mixture was clarified by extracting with $CHCl_3$ and neutralized with 0·1 N-$Ba(OH)_2$. The latter reaction mixture was evaporated to dryness, extracted with boiling water and the extract, cooled, filtered and nearly neutralized with 2 N-H_2SO_4. Neutralization was completed with 0·1 N-H_2SO_4. The volumes of the hydrolysates were adjusted before chromatography so that 1 ml. was equivalent to about 0·3 g. lipid.

Chromatography. A one-dimensional chromatogram was prepared by placing 5 μl. samples of the unknown solutions and suitable reference substances along a line 8 cm. from one end of a 57 cm.-long filter-paper strip (Whatman no. 1). For the preparation of a two-dimensional chromatogram, a 10 μl. sample of the unknown solution was placed in one corner, at a distance of 8 cm. from either edge of a filter-

paper sheet (44·5 × 57 cm.). A second paper, bearing known reference substances superimposed on the unknown spot, was always prepared and run at the same time. Development was carried out at constant temperature (30°) using the descending technique in the apparatus of Dent (1948). The solvent for the one-dimensional chromatograms was either butanol:ethanol:water (4:1:5, v/v) or phenol/water (the phenol was redistilled not more than 24 hr. before use). Two-dimensional chromatograms were prepared using these solvents successively—phenol/water first and along the shorter axis of the paper. The chromatograms were usually run for about 18 hr., by which time the solvent front was a few centimetres from the edge of the paper. Sometimes, in order to obtain better resolution, a longer development time was used and the solvent allowed to run off the paper. When this was done, the bottom edge of the paper was serrated to facilitate even solvent flow. The developed papers were dried for about 18 hr. at room temperature (25–30° approx.) in a chamber fitted with an exhaust fan. As a general treatment for the detection of carbohydrates, etc., on the chromatograms, we dipped the papers first in $AgNO_3$ in aqueous acetone solution, and then, after allowing to dry, in an aqueous ethanolic solution of NaOH (this will subsequently be referred to as $AgNO_3$/NaOH treatment). Our reagents were prepared as described by Trevelyan, Proctor & Harrison (1950) and our method of treatment differed from that of these authors only in that the papers were dipped in, rather than sprayed with, the NaOH reagent. Dipping was found to give a more uniform chromatogram than spraying and did not lead to appreciable spreading of the spots. This treatment, which gave dense, dark-brown spots at room temperature more or less rapidly with reducing sugars, inositol and glycerol, was also found to give, within a few minutes, strongly marked spots with a number of unknown substances in some of the solutions examined. (The latter were detected only faintly or not at all by the ammoniacal $AgNO_3$ reagent of Hough (1950).) On keeping the chromatograms for a few hours, their appearance changed and the components showed clearly as yellow spots, usually with a darker centre, on a light-brown background. To obtain information about the nature of the various components in the unknown solutions and to confirm their identity when an exact correspondence to a known reference substance was observed, we treated some chromatograms with other reagents known to give specific colour reactions with certain types of sugars. We used aniline hydrogen phthalate dissolved in butanol saturated with water (Partridge, 1949), naphthoresorcinol in ethanol solution acidified with H_3PO_4 (Bryson & Mitchell, 1951) and naphthoresorcinol in aqueous ethanol acidified with trichloroacetic acid (Partridge, 1948). The best results were obtained by dipping the papers in the first two reagents but spraying was necessary for the last reagent in order to minimize spreading of the spots.

RESULTS

The undegraded and degraded lipid samples always gave identical qualitative results in these examinations and consequently the former only will be reported here.

Two-dimensional chromatograms prepared from undegraded lipid hydrolysates are represented in Fig. 1. The spots shown are those given by treatment with $AgNO_3$/NaOH. The reactions with other reagents of the substances forming these spots and in some cases their identity are given in Table 1. Vigorous acid hydrolysis of the lipid purified by partition chromatography liberated *meso*inositol, galactose, glucose and glycerol together with a small amount of an unknown substance (spot 4) which appeared from its reactions to be a ketose but which was not identified (Fig. 1*b*). Hydrolysis with 0·01 N-H_2SO_4 yielded small amounts of *meso*inositol, galactose and glucose but no glycerol, while the alkaline hydrolysis gave no galactose or glucose but much *meso*inositol and a little glycerol. In addition, three unknown spots reacting with $AgNO_3$/NaOH were given by both hydrolysates (Fig. 1*d* and *f*). Altogether, five unknown substances were detected in this way since one spot (spot 8 in Fig. 1*d* and *f*) was common to both hydrolysates. The fact that spot 7 (Fig. 1*d*) given by the mild acid hydrolysate reacted with aniline hydrogen phthalate and naphthoresorcinol/H_3PO_4, whereas spot 9 (Fig. 1*f*) given by the alkaline hydrolysate did not, indicates the presence of two different substances in these spots. They occupy closely similar positions on the chromatograms, however, so that one or both of the spots may contain both substances. Neither of the substances designated as *C* and *E* in Table 1 are therefore necessarily homogeneous. Since Resing (1951) hydrolysed his lipid by refluxing with 0·01 N-H_2SO_4 it is possible that his presumed polysaccharide corresponded with one of our substances *B*, *C* or *D*; more likely, since he used only one-dimensional chromatography, it corresponded with a mixture of all three.

Examination of lipid purified by dialysis gave results similar to those for lipid purified by partition chromatography except that quebrachitol was found in the hydrolysates. This, as will be seen later, is present in the lipid as a water-soluble impurity and it appears that it was incompletely removed by dialysis.

Chromatographic examination of hydrolysates of simple fractions prepared from the purified total lipid by precipitation with acetone and ethanol showed that only very small amounts of galactose and glucose were present in the acetone-soluble fraction, and there was no detectable *meso*inositol. Glycerol, presumably derived mainly from triglycerides, was present in this fraction. Both the ethanol-soluble and ethanol-insoluble fractions of the acetone insolubles gave products similar to those given by the total lipid on hydrolysis with 2 N-H_2SO_4, saturated methanolic $Ba(OH)_2$ and 0·01 N-H_2SO_4 respectively. They differed markedly only in that greater amounts of *meso*inositol, and substance *B* were liberated from the alcohol-insoluble than from the alcohol-soluble fraction.

Substances *B–F* appear to be partial breakdown products which are destroyed by vigorous acid hydrolysis. In order to examine these substances further, appropriate partial hydrolysates (1 ml. equivalent to about 0·7 g. lipid) were submitted to one-dimensional paper chromatography. Twenty spots (10 μl.) were placed at 1 cm. intervals along the centre of a line 8 cm. from one end of a filter-paper strip (44·5 × 57 cm.). At each end of this line was placed a further spot of the hydrolysate and a spot containing suitable reference substances to form guide strips. The paper was developed for about 48 hr. with phenol/water. After drying, the guide strips were cut off and treated with $AgNO_3$/NaOH. In this way substances *D–F* were seen to be clearly separated from the alkaline hydrolysate and substances *B* and *C* from the mild acid hydrolysate. Although substance *D* was present in the latter it was not separated from small amounts of glucose and galactose also present. The positions of the various substances on the centre part of the chromatogram were marked by reference to the guide strips and transverse strips were cut to separately contain them. Each substance was eluted by cutting one end of such a strip to a point, immersing the other end in a trough of water contained in a paper chromatographic cabinet, and collecting the drippings. The products of a number of separations were bulked and samples were refluxed with 2N-H_2SO_4 for 24 hr. The identity and purity of the unknown substances and the constituents liberated from them by hydrolysis were examined by one- and two-dimensional chromatography, the chromatograms being treated with the reagents described previously. Table 2 shows the constituents of the hydrolysates identified in this way. A rough indication of their relative amounts based on a visual assessment of the size and intensity of the spots formed is given, but this is recognized to be of limited value particularly since the constituents differed in their resistance to hydrolysis. This was illustrated by the fact that the hydrolysate obtained from substance *B* by refluxing for 1 hr. with saturated methanolic $Ba(OH)_2$ contained a greater amount of glycerol relative to *meso*inositol than did the 2N-H_2SO_4 hydrolysate.

The hydrolysates of lipid from which water-soluble impurities had not been removed, contained up to three substances giving spots with $AgNO_3$/NaOH in addition to those given by the purified lipid hydrolysates (Fig. 1*a*, *c* and *e*). Two of these spots appeared on all the chromatograms and apparently were therefore due to substances resistant to both alkaline and rigorous acid hydrolysis. One of them was identified as quebrachitol (the 2-monomethyl ether of (−)-inositol (Angyal & Macdonald, 1952)) and the other as (−)-inositol itself. A further spot, which was found to be due to fructose, was detected in only the 0·01N-H_2SO_4 hydrolysate. These three substances, together with some glucose and *meso*inositol, were identified in the water-soluble materials recovered from the cellulose-powder column used for purifying the lipid (Fig. 1*g*). It might be expected that since these substances were apparently impurities in the lipid, they would be normally present free in latex serum. Quebrachitol has long been known as a major constituent of latex serum (de Jong, 1906). van Dillen (1922) reported finding galactose, and glucose and/or fructose (characterized as the osazones) in latex dialysate, but only after hydrolysis. It appears that neither *meso*inositol nor (−)-inositol have previously been demonstrated free in latex. We have examined the matter further as follows.

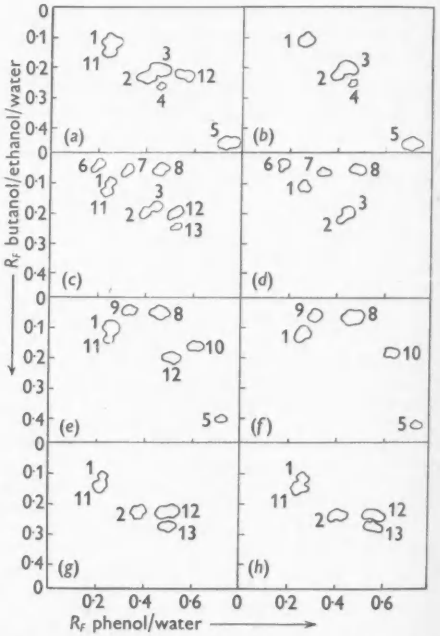

Fig. 1. Two-dimensional chromatograms treated with $AgNO_3$/NaOH. (*a*), (*c*) and (*e*) Hydrolysates of unpurified undegraded lipid (equivalent to 3 mg. lipid), prepared by refluxing with (*a*) 2N-H_2SO_4, (*c*) 0·01N-H_2SO_4 and (*e*) saturated methanolic $Ba(OH)_2$. (*b*), (*d*) and (*f*), hydrolysates of undegraded lipid purified by partition chromatography (equivalent to 3 mg. lipid), prepared by refluxing with (*b*) 2N-H_2SO_4, (*d*) 0·01N-H_2SO_4 and (*f*) saturated methanolic $Ba(OH)_2$. (*g*), water-soluble substances recovered from the cellulose-powder column used for purifying the lipid (amount equivalent to recovery from 6 mg. lipid). (*h*), latex serum prepared by acid coagulation (amount equivalent to 0·02 g. latex). For further details of spots see Table 1.

Table 1. *Further reactions and identity (where known) of substances giving spots with* $AgNO_3$/NaOH *on the two-dimensional chromatograms represented in Fig.* 1

The spot numbers correspond with those in Fig. 1. Identifications were based on the positions on the chromatograms and confirmed by the reactions shown in this table. Spot colours: br., brown; b.g., blue-green; r., red; or., orange; —, no reaction.

Spot no.	Reaction with: Aniline hydrogen phthalate	Naphthoresorcinol/ H_3PO_4	Naphthoresorcinol/ trichloroacetic acid	Identity	Remarks
1	—	—	—	*meso*Inositol	—
2	br.	b.g.	—	Glucose	—
3	br.	b.g.	—	Galactose	—
4	br.	r.	or.	Unknown *A*	Probably a ketose
5	—	—	—	Glycerol	—
6	—	—	—	Unknown *B*	Partial breakdown products. See Table 2
7	br.	b.g.	—	Unknown *C*	
8	—	b.g.	—	Unknown *D*	
9	—	—	—	Unknown *E*	
10	—	b.g.	—	Unknown *F*	
11	—	—	—	(−)-Inositol	—
12	—	—	—	Quebrachitol	—
13	br. (weak)	r.	or.	Fructose	—

Table 2. *Substances liberated on refluxing the lipid partial breakdown products, listed in Table* 1, *with* 2N-H_2SO_4

The partial breakdown products were chromatographically separated from the hydrolysates prepared by refluxing the lipid with 0·01 N-H_2SO_4 or saturated methanolic $Ba(OH)_2$ (details see text). Their constituents, liberated on refluxing with 2N-H_2SO_4, were identified by paper chromatography. The rough relative amounts of these constituents estimated from the size and intensity of the spots obtained are indicated by the number of '+' symbols. The absence of any particular constituent is indicated by the symbol '−'.

Substance hydrolysed	Products: Galactose	Glucose	Unknown *A*	*meso*Inositol	Glycerol
Unknown *B*	−	−	−	+ + +	+ +
Unknown *C*	+ + +	+	−	−	+
Unknown *D*	+ + +	−	+	−	+ +
Unknown *E*	+	+	−	−	+ + +
Unknown *F*	+ + +	+ +	+	−	+

Latex (5 vol.) was coagulated with 0·4N-H_2SO_4 (1 vol.) and the serum was filtered off. Sulphate ions were removed by neutralizing with $Ba(OH)_2$ and most other inorganic ions by passing through a column of Biodeminrolit (Permutit Co. Ltd.). The resultant solution (adjusted in volume so that 1 ml. was equivalent to about 2 g. latex) was submitted to two-dimensional chromatography (Fig. 1*b*). The carbohydrate and polyhydroxy constituents of the serum (at least those reacting with $AgNO_3$/NaOH on a paper chromatogram) were found to consist exclusively of those substances present as water-soluble impurities in the lipid, i.e. glucose, fructose, quebrachitol, *meso*inositol and (−)-inositol. These results were at variance with those of van Dillen (1922) in that we found glucose and fructose in the free form and galactose was absent. We confirmed the presence of free *meso*inositol microbiologically and estimated the amount present to be about 0·021 % of the latex.

DISCUSSION

It has previously been shown (Smith, 1954*b*) that latex lipid prepared by simple solvent extraction contains *meso*inositol and reducing sugars partly in a form closely associated with the phosphatide and partly as water-soluble impurity. The present results have confirmed and extended these observations and appear to show that the carbohydrate and polyhydroxy compounds present in latex lipid in addition to glycerol, can be divided into two classes: (*a*) substances combined with the lipid: galactose, glucose, *meso*inositol and an unknown sugar (apparently a ketose) and (*b*) water-soluble impurities: fructose, quebrachitol, (−)-inositol and further amounts of glucose and *meso*inositol. Confirmation of these conclusions is provided by the fact that all the substances presumed to be water-soluble impurities in the lipid have also been found to be present in a free form in latex serum. Galactose,

the principal combined sugar, has not been found free in the serum.

We have isolated from the purified lipid a number of water-soluble partial-degradation products in which carbohydrates or *meso*inositol are present together with, and presumably in chemical combination with, glycerol. That these substances are bound in the lipid by other than chemical means is unlikely; they must account for a good deal of the glycerol in the phosphatides; they are not known to occur free in nature and they were not removed from the lipid by dialysis or partition chromatography. This, therefore, gives strong evidence that the phosphatides contain both *meso*inositol and carbohydrates in chemical combination. The fact that *meso*inositol is bound to glycerol in one of these products implies the presence of glycerol in the inositol containing phosphatide. This has been found to be so for the inositol containing soya bean phosphatide (Folch, 1947). The isolation of four different partial-degradation products containing carbohydrate and glycerol may mean that one or both of the carbohydrate-containing phosphatides (lecithin and inositol phosphatide) reported previously (Smith, 1954*b*) are not themselves homogeneous. Alternatively, two or more of the partial degradation products may be formed from a common precursor.

It is of interest that Anderson, Lothrop & Creighton (1938) and de Sütö-Nagy & Anderson (1947*a*, *b*) isolated partial-breakdown products of the phosphatides of the tubercle bacillus and found among them substances containing glycerol combined with mannose or *meso*inositol. These substances, which may be similar to those described in the present paper, also contained phosphoric acid.

SUMMARY

1. Paper-chromatographic examination has shown that latex lipid prepared by simple solvent extraction without special purification, contains (*a*) in a combined form—galactose, glucose, an unknown sugar (probably a ketose) and *meso*inositol; and (*b*) as water-soluble impurities—glucose, fructose, *meso*inositol, quebrachitol and (−)-inositol. The latter substances were all found to be present, in a free form, in a sample of latex serum. They were removed from the lipid by partition chromatography on cellulose or, less efficiently, by dialysis of an aqueous suspension.

2. Partial hydrolysis of the purified lipid led to the formation of five substances which were separated by paper chromatography and which on further hydrolysis yielded glycerol and either *meso*inositol or one or more of the above carbohydrates. This is regarded as giving strong evidence that both *meso*inositol and carbohydrate are chemically combined in the lipid.

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Some Serological and Chemical Studies on Materials Extracted from an Amylolytic Streptococcus from the Rumen of the Sheep

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In recent years numerous types of amylolytic organisms have been shown to be present in the digestive tracts of animals (Van der Wath & Myburgh, 1948; Baker, Nasr, Morrice & Bruce, 1951; Masson, 1951; Huhtanen & Gall, 1953), and MacPherson (1953) has lately reported the isolation of a number of strains of amylolytic streptococci from the rumens of sheep fed on a variety of diets. The amylases of one of these strains of streptococci (BSO7) and of a rumen strain of *Clostridium butyricum*, and an intracellular, amylopectin-type polysaccharide from a pig caecum strain of *Cl. butyricum* have been described by Hobson & MacPherson (1952) and Hobson & Nasr (1951). Continuing the investigation of these streptococci we here report some findings on serologically active materials from the strain BSO7. Encapsulation appears to be common in rumen bacteria *in vivo* (Hobson & MacPherson, 1953*a*) so that besides their bacteriological interest, capsular polysaccharides may have some significance in ruminant nutrition as they represent one of the products of the breakdown of feeding-stuff polysaccharides by rumen micro-organisms.

The Lancefield (1933) and Fuller (1938) methods of extracting antigens from streptococci were found to give materials which were serologically not entirely comparable, although either method is commonly used for grouping pathogenic streptococci. Examination of the two extracts showed them to consist of protein and polysaccharide. In a number of cases a polysaccharide has been shown to confer the specificity for the organism (Kendall, Heidelberger & Dawson, 1937; Heidelberger, Kendall & Scherp, 1936; Mirick, Thomas, Curnen & Horsfall, 1944; Julianelle, 1926; Morgan, 1937), and since MacPherson & Oxford (1952) have shown that the capsules of these bacteria reacted with specific antisera, it appeared likely that a type-specific polysaccharide was part of the capsule, especially as other workers have shown that the majority of bacterial capsules, with the exception of those of some *Bacillus* spp. (Ivánovics & Bruckner, 1938) are composed mainly of polysaccharide. Attention was, therefore, directed to the preparation of polysaccharides from the medium in which the bacteria had grown (the 'metabolic liquid') and from the bacterial capsules. A preliminary report of this work has already been given (Hobson & MacPherson, 1953*b*).

EXPERIMENTAL AND RESULTS

Methods and materials

Analytical methods. Before analysis the preparations were dried to constant weight *in vacuo* at 60° over P_2O_5. In all determinations standard solutions were included along with the unknowns to calibrate the reagents.

Reducing sugars were determined using Somogyi's (1952) micro copper reagent in conjunction with a colorimetric reagent (Nelson, 1944) calibrated against glucose. A correction was made for the small loss of sugars during the hydrolysis (see Pirt & Whelan (1951) for glucose; similar results were found with galactose and rhamnose).

Rhamnose was measured by a microspectrophotometric method (Dische & Shettles, 1948) after standardizing against rhamnose monohydrate.

Glucosamine was determined with the reagents of Elson & Morgan (1933) calibrated with glucosamine hydrochloride and using a photoelectric colorimeter. *Total nitrogen* was determined by a micro-Kjeldahl method. *Uronic acids* were determined by the modification according to Bray, Humphris, Thorpe & Wood (1952) of the method of Hanson, Mills & Williams (1944). The reagents were calibrated against a mixture of galacturonic acid, galactose and rhamnose in the approximate proportions of the polysaccharide to obviate any effects of the sugars on the reagents (Hanson *et al.* 1944). *The ash content* was determined by incinerating a little of the material, in a platinum boat, to constant weight in a micro-muffle. *Total phosphorus* was determined by the method of Allen (1940) after combustion of the material with perchloric acid. *Acid hydrolysis* was carried out by heating the material (concentration approx. 0·5 mg./ml.) with 1·5 N-H_2SO_4 for the stated time in a boiling-water bath in a lightly stoppered flask. For reducing-sugar determinations the hydrolysate was neutralized with 5 N-NaOH and diluted to a known volume.

Paper chromatography. The acid hydrolysates were neutralized with $BaCO_3$, filtered, shaken alternately with small portions of cation- and anion-exchange resins (Amberlite IR 100H and IR 4B; Rohm and Haas Co., Philadelphia, U.S.A.) and evaporated to dryness. The residue was dissolved in an appropriate small volume of water and a suitable amount applied to the starting line of a strip of Whatman no. 1 paper along with reference sugars. The chromatograms were developed with the top layer of a benzene:butanol:pyridine:water mixture (1:5:3:3, v/v) for 24 hr. at room temp. After drying, the sugars were

coloured by spraying with a benzidine reagent (Bacon & Edelman, 1951). Other reagents were used to try to demonstrate the presence of ketoses or to colour hexosamines.

Negative staining of capsules by the wet indian ink method. The films were prepared as described by Mackie & McCartney (1950) and examined by phase contrast illumination. Before making the films the pH of the sample was adjusted to 7 to prevent agglutination of the ink particles. High salt concentrations were also found to agglutinate the ink.

Preparation of sera. The sera were obtained from rabbits after a course of intravenous injections of formalized suspensions of the organisms over a period of some weeks as described by MacPherson (1953). Lancefield's group D serum was obtained from Burroughs, Wellcome and Co., London. The homologous immune sera were standardized before use by agglutination of a standard cell suspension.

Precipitin tests. Serial dilutions of the appropriate antigenic material in neutral solution were prepared and layered on top of the immune serum in Dreyer's agglutination tubes, and after incubation for 30 min. at 37° were examined for interfacial precipitate. The results obtained in this way were confirmed by complement-fixation tests.

Precipitin reactions in gels. The method used is described by Oudin (1952), a mixture of agar and BSO 7 immune serum being used as internal reactant, the external reactants being solutions of the purified polysaccharides.

Culture of the organism BSO 7

(*a*) *For Fuller extraction.* An 18 hr. nutrient broth culture (1 ml.) was inoculated into 4 l. of medium containing 1% Lab Lemco (Oxo Ltd., London), 1% peptone (Evans Medical Supplies Ltd., London), 0·5% NaCl (A.R.), 0·5% glucose (A.R.) which was incubated at 40° for 48 hr. The medium, with the exception of the glucose, was sterilized by autoclaving for 15 min. at 121°, and the glucose, sterilized by filtration, added as a 50% solution after cooling. The initial pH of the medium was 6·8.

(*b*) *For Lancefield extraction.* An 18 hr. culture (1 ml.) was inoculated into 4 l. of medium containing 1% peptone, 0·5% yeast extract (Difco, dehydrated. Difco Co., Detroit, Michigan, U.S.A.), 0·5% Lab Lemco, 0·5% Bacto-tryptone (Difco), and 0·5% glucose and incubated for 48 hr. at 40°. The medium was sterilized as before, the initial pH being 7·6.

(*c*) *For preparation of polysaccharides from the metabolic liquid and capsules.* The medium used was as in (*a*).

Preparation and analysis of the extracts

Fuller extraction. The procedure used was similar to that described by Fuller (1938), but as larger quantities of cells were used it was modified as follows. The cells were removed from a 48 hr. culture on a Sharples 'Supercentrifuge' and after being washed twice with sterile water on an angle centrifuge the sediment was suspended in formamide (B.D.H. Laboratory Reagent; 40 ml.) and heated at 150° in an oil bath for 15 min. After cooling, acid-alcohol (100 ml. containing 95 ml. ethanol, 4 ml. water, 1 ml. conc. HCl) was added with stirring and the precipitate removed on the centrifuge. To the supernatant liquid, acetone (200 ml.) was added and the precipitate centrifuged off after 10 min., dried by trituration with acetone and finally in a vacuum desiccator over P_2O_5.

Lancefield extraction. The washed cells were prepared from 4 l. of a 48 hr. culture, as for the formamide extraction. The organisms were suspended in 0·05 N-HCl (80 ml.) containing a few drops of congo red (0·2% aqueous solution). 1 N-HCl was then added until the indicator turned slate blue. The whole was then immersed in a boiling-water bath for 10 min., when a pink colour developed, and, after rapid cooling, was centrifuged. The sediment was discarded. After addition of a few drops of phenol red (B.D.H. indicator) the clear supernatant was neutralized with 1 N-NaOH and centrifuged. The supernatant solution was usually used for precipitin tests without further treatment. However, in order to obtain a solid for analysis the polysaccharide was precipitated by the addition of acetone (2 vol.) and dried in acetone and *in vacuo* over P_2O_5. Precipitation of the material did not appear to alter the serological reactions. For precipitin tests a known amount of the material was dissolved as far as possible in physiological saline (0·85% NaCl) and any insoluble residue centrifuged off, dried, and weighed to give the amount of material in solution.

Determination of the amino acid content of the Fuller extract. To determine whether the nitrogen content of the Fuller extract was due to protein or hexosamine, a determination of the α-amino-nitrogen content of a hydrolysate of the material was made. A portion of the material was hydrolysed in a sealed evacuated tube in 6 N-HCl for 24 hr. at 105°. The HCl was removed by distillation *in vacuo* and the residue diluted to a known volume. The α-amino nitrogen was determined by the ninhydrin/CO_2 method (Van Slyke, Dillon, MacFadyen & Hamilton, 1941), the pH of the buffer used being 4·7 and the volume of solution 3 ml. Two-dimensional chromatograms of the oxidized hydrolysate were developed in aqueous phenol and collidine solvents, the phenol in an atmosphere of coal gas. The amino acids were coloured by spraying with ninhydrin.

The results of analysis and the precipitin reactions of both extracts are shown in Tables 1 and 2. The sugars found in each hydrolysate were the same. The nitrogen content of the formamide extract was due to both protein and hexosamine-containing polysaccharide. The amino acids found on chromatograms of this material were aspartic and glutamic acids, glycine, threonine, alanine, histidine, proline, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine, arginine and lysine. Serine was probably present but the resolution of that and the glutamic acid was not complete. Hexosamine was also shown on these chromatograms. α:ε-Diaminopimelic acid, which was found in a number of micro-organisms (Work & Dewey, 1953), was not present in this extract. Synge (1953) has examined four strains of amylolytic rumen streptococci and found this acid to be absent from all. While the formamide extract gave a weak reaction with group D antiserum, no reaction could be detected with the acid extract. Both showed positive reactions with the homologous immune serum, but while the acid extract did not react with homologous serum after the solution had been stored at room temperature for 48 hr., the reaction of the formamide extract was unaltered after a similar period.

Table 1. *Analysis of serologically active materials from an amylolytic rumen streptococcus* BSO 7

Preparation	Yield (mg.)*	Sugars (as glucose) after hydrolysis† (%)	Ash (%)	N (%)	Uronic acid (%)	Rhamnose (%)	Sugars in hydrolysate‡
Fuller (formamide) extract	101·1	34·9	—	8·6§	‖	—	Glucose, galactose, rhamnose, ribose, hexosamine
Lancefield (acid) extract	309·5	41·0	—	4·3	‖	13·9	Glucose, galactose, rhamnose, ribose, hexosamine
Metabolic liquid polysaccharide (18 hr. culture)	284·0	37·4	—	3·8	‖	4·4	Glucose, galactose, rhamnose, ribose
Metabolic liquid polysaccharide (48 hr. culture)	1400·3	11·2	74·7	0·6	6·3	1·5	Glucose, galactose, rhamnose, xylose
Capsular polysaccharide (48 hr. culture)¶	113·4	58·2	7·2	0·7	22·0	17·6	Galactose, rhamnose, glucose (trace)

* In 5 l. of culture.
† For 6 hr. in 1·5 N-H_2SO_4 at 100°; 3 hr. hydrolysis gave slightly lower results.
‡ Detected by paper chromatography.
§ 69·2% of this was α-amino nitrogen.
‖ Qualitative test showed positive result.
¶ Phosphorus content, 0·2%.

Table 2. *Precipitin titres of serologically active materials from an amylolytic rumen streptococcus* BSO 7

Preparation	Precipitin titre (mg./ml. × 10^5)*	
	BSO 7 immune serum	Lancefield group D serum
Fuller (formamide) extract	1200	12 000 (or rather less)
Lancefield (acid) extract	120·6	No reaction
Metabolic liquid polysaccharide (18 hr. culture)	71·0	55 800
Metabolic liquid polysaccharide (48 hr. culture)	1404 (800†)	10 800
Capsular polysaccharide (48 hr. culture)	35·7 (13·0†)	453 000

* Least concentration of preparation to give a positive precipitin reaction.
† Complement-fixation titration.

Schmidt (1952) in his studies of group A streptococcal polysaccharide digested the protein in the cells with pepsin. An attempt was made to remove the protein from an acid extract prepared from *Streptococcus faecalis* (N.C.T.C. no. 775) by digestion with crystalline trypsin (Armour Laboratories, Lindsay Street, London, E.C. 1) but analysis of the resulting material showed no improvement in the nitrogen, reducing-sugar content (after hydrolysis), or precipitin titre, over the untreated extract, so the method was not further investigated.

Preparation of a polysaccharide from the metabolic liquid. Since it was possible to demonstrate the presence of a serologically active material in the cell-free supernatant from liquid cultures (MacPherson, 1953) of the streptococci, it was thought that a polysaccharide uncontaminated by intracellular material might easily be obtained from this. The method used was a modification of that of Kendall *et al.* (1937), whose method has also been used by Loewenthal (1938) for the isolation of a polysaccharide from haemolytic streptococci. A young culture was used, as this would be less likely to contain material from autolysed cells.

The cells were removed from 1 l. of an 18 hr. culture (see above) by centrifuging at +1° (all subsequent operations were carried out at +1°). The sediment was washed with two lots of 0·1 M phosphate buffer (pH 7·0; total 130 ml.) and the washings added to the original supernatant. To the clear liquid (approx. 1 l.) ethanol (2 vol.) was added and the whole stored at +1° for 48 hr. The precipitate was removed on the centrifuge and dissolved in water (200 ml.) containing hydrated sodium acetate (10 g.) and glacial acetic acid (5 ml.). The solution was divided, for convenience, into two portions and each deproteinized by shaking with successive lots of a mixture of $CHCl_3$ (25 ml.) and butanol (2·5 ml.) (Sevag, 1934). The final aqueous solutions were combined and recentrifuged and to the clear liquid 2 vol. of ethanol were added. After storing for 18 hr. the fine precipitate was removed on the centrifuge, dissolved in water, diluted to 50 ml. and centrifuged. A portion (1 ml.) was removed for precipitin tests, and the polysaccharide quantitatively precipitated from the remainder of the supernatant by addition of ethanol (2 vol.) and dried in acetone as before. The results of analysis and the precipitin titrations are given in Tables 1 and 2.

Preparation of the capsule polysaccharide. Morgan (1937) described several organic solvents which would dissolve polysaccharide from a number of organisms. Of these, ethylene and diethylene glycols appeared to be the best. Seastone (1939) removed the capsules from group C haemolytic

streptococci with phosphate buffer, pH 7·0, and with 1% aqueous chloroform. All these reagents were tried on small portions of washed cells of *streptococcus* BSO7. Wet indian ink preparations showed no reduction in capsule size after treatment, and no precipitate was formed on addition of excess ethanol to the supernatant liquids. The streptococcus cells were then heated in sodium acetate-acetic acid solutions of different pH and it was found that a few minutes heating at 100° in a solution of about pH 2 removed the capsules, without apparently damaging the cells. (This method is similar to that of Porges (1905) who used hot 0·25N-HCl for removing the capsules of *Klebsiella pneumoniae*.) The method was adopted for the large-scale preparation of the capsule polysaccharide.

The cells from 5 l. of a 48 hr. culture were removed on the Sharples centrifuge and washed three times with distilled water on an angle centrifuge. To the wet suspension of cells (100 ml.; concentration of organisms approx. 10^{10}/ml.) 0·2M sodium acetate (100 ml.; hydrated salt made up in 85% (v/v) aqueous acetic acid) and Tween 80 (Honeywell and Stein Ltd., London; 0·2 ml.) were added and the whole was heated in a boiling-water bath with frequent shaking. At intervals small samples were removed, neutralized with 5N-NaOH and examined in wet indian ink preparations. The decrease in size of the capsules on heating is shown in Table 3. In the medium the average diameter of the cells and capsules was 2·4 μ.; after washing and centrifuging the diameter was 2·1 μ.

After 25 min. the majority of the cells were decapsulated, their appearance before and after acid treatment being shown in Fig. 1*a*, *b*. The suspension was rapidly cooled, centrifuged and the sediment washed with water. The supernatant and washings (300 ml.) were divided into two portions and deproteinized by shaking with $CHCl_3$ and butanol as before. To the final aqueous layer ethanol (2 vol.) was added and after storing at +1° for 48 hr. the precipitate was removed on the centrifuge and dissolved in water (50 ml.) to give a clear, viscous solution. This was centrifuged, a portion removed for precipitin tests and the polysaccharide quantitatively precipitated from the remainder by addition of a little NaCl and ethanol (150 ml.). The polysaccharide was dried by trituration with acetone and in a vacuum desiccator over P_2O_5 to give a white powder entirely soluble in water to a clear, viscous solution.

A polysaccharide was prepared by the method previously described after filtering and concentrating the metabolic liquid (5 l.) from the above culture to approx. 650 ml. *in vacuo*. The qualitative analysis of this material was similar to that previously prepared from the 18 hr. culture, but it contained a very large amount of ash (Table 1). This was probably mainly caused by evaporation of the liquid to a small volume before the first precipitation.

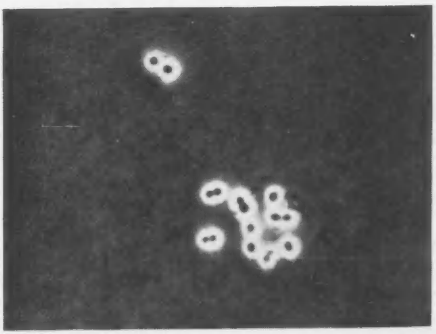
Attempted identification of the uronic acid. Various spectrophotometric methods were tried without success to identify the uronic acid present in the whole polysaccharides and hydrolysates. Finally, portions of the metabolic liquid polysaccharide (48 hr. culture, above) were hydrolysed for 6 and 11 hr. in 1N sulphuric acid. Glucuronolactone and galacturonic acid controls were heated for the same times in acid solution. After cooling, the solutions were diluted and barium chloride was added in sufficient amount to precipitate most of the sulphate ions. The precipitate was removed on the centrifuge,

Table 3. *Removal of capsules from an amylolytic rumen streptococcus* BSO7 *with sodium acetate-acetic acid*

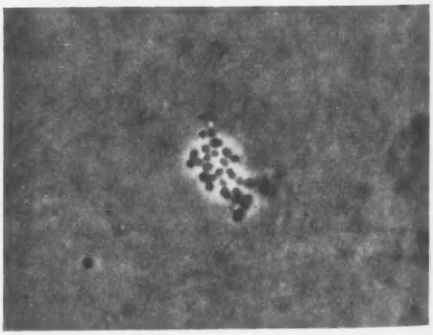
Time of heating in 100° water bath (min.)	Overall diameter of cell + capsule (μ.)*
0	2·1
10	2·1
20	1·4
25	1·0†

* Measured with calibrated micrometer eyepiece across shortest diameter.

† Cells not visibly damaged.



a



b

Fig. 1. Removal of capsules in sodium acetate-acetic acid. Wet indian ink preparations. Phase-contrast illumination. Magnifications, ×1600. (*a*) Pure culture of rumen streptococcus (BSO7) with capsules outlined by indian ink particles. (*b*) The same culture after removal of the capsules with sodium acetate-acetic acid.

washed, and the washings and supernatant liquid neutralized with 1N sodium hydroxide and evaporated to dryness *in vacuo*. 5N sulphuric acid (3–4 drops) was then added to each residue to acidify and moisten it and each was then extracted with two 10 ml. portions of pyridine at 100° for 10 min. (Malpress & Morrison, 1949). The extracts were evaporated to syrups at 40° or below *in vacuo* and the syrups were further dried as far as possible in a vacuum desiccator. The polysaccharide residue in the initial hydrolysate was very brown and gave a long streak on the chromatograms before spraying with a saturated solution of *o*-phenetidine and trichloroacetic acid in ethanol. Further pyridine extracts were, therefore, treated in aqueous solution in a boiling-water bath with active charcoal, filtered and evaporated to dryness to give a clear 'glassy' residue. Chromatograms of the hydrolysates together with the galacturonic acid and glucuronolactone controls were developed in pentanol:pyridine:water (7:7:6, v/v) and the top layer of a butanol:acetic acid:water mixture (4:1:5, v/v). (On some chromatograms in the latter solvent mannuronolactone was included.) On all the chromatograms the polysaccharide showed an initial spot about the position of the galacturonic acid controls with streaking below due to the presence of degradation products. No spots corresponding to the faster-moving glucuronic acid or lactone, or mannuronic acid could be detected. It seems, therefore, that the uronic acid present is galacturonic, although the presence of degradation products makes the chromatograms difficult to interpret.

The results of analysis and precipitin tests are shown in Tables 1 and 2. Precipitin tests were also carried out in agar-serum gels, the external reactant being the capsular polysaccharide (4·5 mg./ml. in water) and the polysaccharide from the metabolic liquid (13·6 mg./ml.). The capsular polysaccharide diffused farthest into the gel forming a single band of precipitate with a sharp leading edge and a rather diffuse upper part. The polysaccharide from the metabolic liquid formed a diffuse band which travelled about half the distance of the capsular polysaccharide and the front was not so well defined. Using a mixture of the two polysaccharides as external reactant two precipitate bands were formed in the gel, the distances travelled corresponding with those of the separate solutions.

DISCUSSION

Two methods are in general use for serological classification of streptococci. These are based on group and type specificity. In Lancefield's classification (Lancefield, 1928, 1933) streptococci having a common antigen are placed in the same group designated by one of the letters A to N. Intestinal streptococci have, in general, been found to belong to group D. Groups A and D streptococci have been subdivided into types by Griffith (1934) and by Sharpe & Shattock (1952), respectively. These types possess, besides the common group antigen, a type-specific antigen which has been associated with polysaccharide in the capsule (Kendall *et al.* 1937). The group-specific substance has been shown to be intracellular and to be either protein or polysaccharide in nature (Heidelberger & Kendall, 1931, 1936).

In the experiments described, the Lancefield and Fuller methods, although intended to extract similar antigens, gave products with different serological reactions (Table 2), but of similar chemical composition. The great difference between the reagents, and the somewhat drastic procedures involved in the extractions, could be possible reasons for the discrepancies in the reactions of the products.

The polysaccharide isolated from the metabolic liquid of an 18 hr. culture had a lower nitrogen content than the acid and formamide extracts and a higher precipitin titre with the homologous-type serum, but in higher concentration it gave a reaction with the group serum. This was to some extent non-specific, as solutions of higher concentration gave a reaction with normal rabbit serum. The capsular polysaccharide reacted in extremely low concentration ($35{\cdot}7 \times 10^{-5}$ mg./ml.) with the homologous-type serum and only in high concentration (4·5 mg./ml.) gave a faint reaction with the group D specific serum. No reaction was given by the polysaccharide in this concentration with normal serum. No conclusions can be drawn about the group-specific material in the organisms except to suggest that it is probably intracellular as it is found in the formamide extract of whole cells and in a greater amount in the metabolic liquid polysaccharide of the old culture (48 hr.) than of the 18 hr. culture, and the former would be expected to contain more autolysed cells. The injection of capsular polysaccharide into rabbits to see if it acts as a complete antigen has not been tried.

The results of the precipitin reactions in gels suggest that the metabolic liquid polysaccharide is of somewhat higher molecular weight than the capsular polysaccharide, or is linked to protein not present in the capsular polysaccharide, as it diffused a smaller distance into the gel. As each solution formed only one precipitate band it may be assumed that there is only one constituent in each preparation capable of giving a precipitin reaction with the BSO 7 immune serum. This is supported by the results of ultracentrifuge examination, reported in the Addendum, which show that the capsular polysaccharide is essentially homogeneous in molecular size.

Galactose, rhamnose and uronic acid were common to the sugar constituents of the hydrolysates of the preparations. Glucose was also present in a similar concentration in the hydrolysates of the acid and formamide extracts and the two polysaccharides isolated from the metabolic liquid. This glucose may be present in an intracellular polysaccharide (it has been found in hydrolysates of whole group A streptococci by Consden & Stanier (1952) and the metabolic liquid might be expected to contain material from some autolysed cells), or it may be due to some extent to adsorption from the medium on to the precipitates. The latter is possible, as traces of arabinose were found in hydrolysates of a metabolic liquid polysaccharide and control experiments showed that small amounts of this sugar were present in the medium together with the glucose. The small amount of xylose in the metabolic liquid (48 hr. culture) polysaccharide is at present unexplained, although it has been found in some bacteria (Belozerskiĭ, 1952; Hofman, 1953). The presence of ribose in the acid and formamide extracts and traces in the metabolic liquid (18 hr. culture) polysaccharide suggest that nuclear material is contained in these preparations. No ketose sugars were found in hydrolysates, but some hexosamines could be detected in chromatograms of the hydrolysates of the acid- and formamide-extracted polysaccharides. The purified capsular polysaccharide is composed of galactose, rhamnose, and a uronic acid, probably galacturonic acid, with a trace of glucose. Like some other polysaccharides containing uronic acids (see, for example, Hough, Jones & Wadman, 1952) the initial rate of acid hydrolysis was rapid, but after 3 hr. in 1·5N sulphuric acid only a slow increase in reducing sugars with time of heating was found (after correcting for the slight destruction of the sugars by acid). This was not increased by the use of 3N acid, which greatly increases the rate of destruction of the sugars. Galactose, rhamnose and galacturonic acid each have a lower reducing power than glucose with the reagents used, and this and the slow rate of hydrolysis would account for the apparently low reducing-sugar content after hydrolysis.

A purification of the type-specific material from this streptococcus has thus been achieved and it has been shown to be a capsular polysaccharide in conformity with the results obtained with other bacteria. Schmidt (1952) obtained a polysaccharide from group A streptococci containing 26·1% glucosamine, 60·8% rhamnose and 2·6% nitrogen which reacted in high dilution with group A serum. Consden & Stanier (1952) reported glucose, rhamnose, uronic acid and hexosamine in unpurified hydrolysates of group A streptococci. Hofman (1953) found galactose, ribose, rhamnose and xylose in hydrolysates of *Nitrosomonas*. Salton (1952) found glucose, galactose, rhamnose, and hexosamine in cell wall preparations of *Streptococcus faecalis*. Polysaccharides containing uronic acids have been isolated from the pneumococci and the polysaccharide from this amylolytic streptococcus resembles that of type II pneumococcus (Kent, 1952) which contains glucose, rhamnose and glucuronic acid.

SUMMARY

1. Materials extracted from an amylolytic rumen streptococcus by the Lancefield (1933) and Fuller (1938) methods have been compared for serological activity and chemical composition. Similar tests have been made on polysaccharides prepared from the metabolic liquid.

2. A method has been developed for removing the capsules from these streptococci.

3. The capsular polysaccharide has been shown to possess a high type-specific serological activity.

4. An analysis of the capsular polysaccharide has been carried out and its main constituents have been shown chromatographically to be galactose, rhamnose and a uronic acid, probably galacturonic, the analysis being; reducing sugars, as glucose, after acid hydrolysis, 58·2%; N, 0·7%; P, 0·2%; ash, 7·2%; uronic acid, 22·0%; rhamnose, 17·6%.

The authors wish to express their thanks to Dr A. E. Oxford for helpful discussions during the course of this work. They are also indebted to Dr F. H. Malpress for discussion of the method of pyridine extraction of sugars, to Mr S. Mann for help in preparing sera, and to Miss M. G. Garvock and Mr J. Wood for technical assistance.

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ADDENDUM

A Physico-Chemical Examination of the Capsular Polysaccharide from an Amylolytic Sheep Rumen Streptococcus

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(*Received* 28 *October* 1953)

The capsular polysaccharide from the amylolytic rumen streptococcus BSO7 described in the preceding paper has been examined in the ultracentrifuge, and in addition its partial specific volume and limiting viscosity number* have been determined.

METHODS

Solution. The dried polysaccharide was dissolved in a buffer containing 0·2 M-NaCl, 0·0633 M-Na_2HPO_4 and 0·0033 M-KH_2PO_4 (pH 7·8). A 1% (w/v) solution was diluted by weight as required and used for all measurements. (Weights were corrected for 7·2% of ash present in the material.)

Sedimentation. Rates of sedimentation were determined using an electrically driven 'Spinco' ultracentrifuge (Specialized Instruments Corporation, Belmont, California, U.S.A.) at rotor speeds of 1000 rev./sec. The pressure in the rotor chamber was less than 1 μ. Hg, and the temperature rise in the rotor itself was about 0·6°/hr. (the refrigerating system was not utilized). Movement of the boundary was followed using a modified Philpot-Svensson optical system. The magnification of this lens system was 2·22, constant over the whole field, and the average distance of the reference line from the axis of rotation was taken as 5·730 cm. (at 1000 rev./sec.). Measurement of the rates of sedimentation were made at several concentrations, the distance through which the boundary had moved being measured, to 0·02 mm., directly from the photographic plate with a travelling microscope. Sedimentation constants were evaluated from the slope of $d \log_{10} x/dt = S/2{\cdot}303$, x being measured in cm. from the centre of rotation and t in seconds from an arbitrary origin, and converted to water at 20°.

Viscosity. An Ostwald type viscometer was used, the essential dimensions being as follows: diameter of capillary, 0·5 mm.; length of capillary, 12·5 cm.; working volume, 2 ml.; mean pressure head, 13·0 cm.; efflux time for solvent, 83·8 sec.; temp. 22·5°. The viscosity number (η_{sp}/c) was determined at several concentrations (where $\eta_{sp} = \eta_{rel} - 1$, and η_{rel} = solution/solvent viscosity ratio), and the limiting

* The nomenclature used in this paper is in accordance with the 'Report on Nomenclature in the Field of Macromolecules of the International Union of Pure and Applied Chemistry', reported in *J. Polym. Sci.* (1952), **8**, 257.

viscosity number $[\eta]$ obtained from the graph of η_{sp}/c against c, as

$$[\eta] = \lim_{c \to 0} (\eta_{sp}/c).$$

Partial specific volume. The densities of solutions of several concentrations were determined using a pyknometer, and the partial specific volume ($\bar{v}$) calculated from the equation (Svedberg & Pedersen, 1940)

$$(1 - \bar{v}\rho) = (1 - w/m)\, dm/dw,$$

where ρ is the density of a solution for which the weight fraction of solute is w and the mass of a given volume m; dm/dw was determined from the linear graph of m against w.

RESULTS

In 0·2 M sodium chloride buffered with phosphate at pH 7·8 the presence of only one component in the polysaccharide was apparent from the sedimentation determinations. The boundaries appeared symmetrical and, for higher concentrations, sharp. Typical sedimentation diagrams are shown in Fig. 1. The values of the sedimentation constants (S_{20}) for three concentrations are recorded in Table 1. Owing to lack of material it was not possible to examine a wider range of concentrations, but a marked dependence of sedimentation constant on concentration was observed. The limiting value of S_{20} was determined using a method suggested by Jullander (1945). In this method S_{20} and $S_{20} \times \eta_{rel}$ are both plotted against c on the same graph and since

$$\lim_{c \to 0} S_{20} = \lim_{c \to 0} S_{20} \times \eta_{rel}$$

the two functions approach the same limiting value and hence define it more exactly. Fig. 2 shows these results and the limiting value of S_{20} is seen to be $3{\cdot}25 \pm 0{\cdot}2 \times 10^{-13}$.

Experimentally determined values of η_{sp}/c are also given in Table 1, together with the extrapolated value of the limiting viscosity number, 3·75. The value for the partial specific volume of the material was found to be 0·60.

The high limiting-viscosity number suggested that the molecules were very asymmetrical, and this figure was used to obtain the approximate axial ratio ($1/d$) and hence the frictional ratio (f/f_0), as a preliminary to the molecular-weight calculation. (This procedure assumes that a value of $f/f_0 > 1$ indicates a deviation from spherical shape when f, the actual molar frictional coefficient $= M(1 - \bar{V}\rho)/S$, and f_0, the molar frictional coefficient of a spherical, non-solvated particle $= 6\pi\eta N\,(3M\bar{V}/4\pi N)^{\frac{1}{3}}$. f/f_0 can then be related to the axial dimensions $1/d$ of the equivalent ellipsoid, where $1/d =$ length (1) of major axis/diameter (d) of equatorial axis.)

The axial ratio ($1/d$) was calculated by the use of Simha's equation (Simha, 1945).

$$\eta_{sp}/\phi = 0{\cdot}233\,(1/d)^{1{\cdot}698},$$

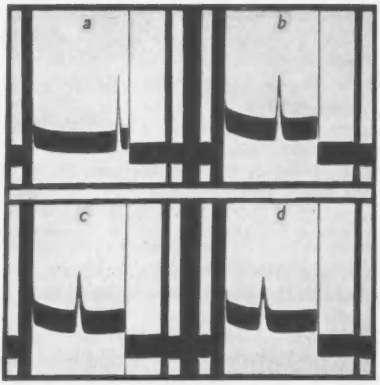

Fig. 1. Sedimentation diagrams for the capsular polysaccharide. Speed, 1000 rev./sec. Solvent, 0·2 M-NaCl buffered with phosphate to pH 7·8. Concentration, 0·37 g./100 ml. Times of exposures (*a*) 37 min., (*b*) 129 min., (*c*) 142 min., (*d*) 177 min., from starting the centrifuge.

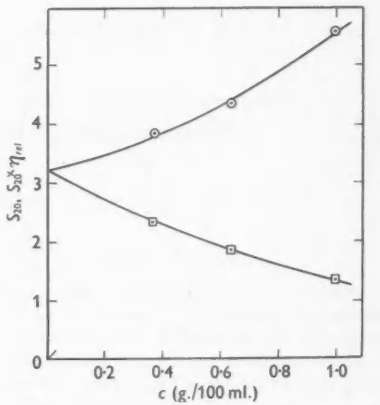

Fig. 2. Determination of limiting value of S_{20} by the method of Jullander (1945). For explanation see the text.

Table 1. *S_{20} and η_{sp}/c values at different values of c for the capsular polysaccharide*

Concentration, c (g./100 ml. solution)	Sedimentation constant $(S_{20}) \times 10^{13}$	Viscosity number (η_{sp}/c)
1·00	1·34	—
0·64	1·87	4·0
0·37	2·34	—
0·34	—	3·9
0·17	—	3·8
0·00	[3·25]*	[3·75]†

* See text and Fig. 2. † By extrapolation.

when $20 \leqslant 1/d \leqslant 100$ and ϕ is the volume fraction of solute. For the concentration units used (g./100 ml. solution) this expression reduces approximately to

$$[\eta]\ 100 = 0{\cdot}233\ (1/d)^{1{\cdot}698},$$

and from this the axial ratio $(1/d)$ was found to be 77. This value represents a frictional ratio (f/f_0) of 3·5, assuming the particles to be prolate spheroids, and using the data of Herzog, Illig & Kudar (1933). (Any hydration effect has been ignored.) The molecular weight M was then calculated from the equation

$$M = 2{\cdot}45 \times 10^{22}\, \overline{V}^{\frac{1}{2}} (f/f_0)^{\frac{3}{2}} [S/(1 - \overline{V}\rho)]^{\frac{3}{2}}.$$

The frictional ratio (f/f_0) of 3·5 gave a value of about 90000. If the molecule was assumed to be spherical and unsolvated (i.e. $f/f_0 = 1$), the value of 14000 was obtained.

DISCUSSION

The results show that the polysaccharide is not obviously polydisperse and the high limiting-viscosity number, and the marked dependence of the sedimentation constant on concentration suggest that the molecules are very asymmetrical. (Factors causing a dependence of sedimentation constant on concentration have been discussed by Kinell & Rånby (1950), and Ogston & Woods (1953).) The value of 90000 for the molecular weight calculated using the frictional ratio obtained from the viscosity measurements is regarded as being of the correct order of magnitude, whilst the figure of 14000 obtained assuming the molecule is spherical represents the minimum value. The effect of hydration could, however, reduce the higher figure. Few measurements have been made on similar polysaccharides from other bacteria, but Record & Stacey (1948) have reported molecular weights of 171×10^3, 540×10^3, and 141×10^3 for pneumococcus types I, II and III polysaccharides. Results obtained with other polysaccharides have been reviewed by Greenwood (1952).

SUMMARY

1. The capsular polysaccharide from an amylolytic rumen streptococcus has been found to be homogeneous in the ultracentrifuge.

2. The material possesses a high limiting-viscosity number and hence a very extended shape in solution (the frictional ratio $(f/f_0) = 3{\cdot}5$).

3. The molecular weight of 90000 calculated using the frictional ratio obtained from the viscosity measurements is regarded as being more correct than that calculated assuming a spherical molecule.

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The Distribution and Metabolism of Phosphorus Compounds in Normal and Demyelinating Nervous Tissue of the Chicken

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Several studies have been reported on the composition and metabolism of the phospholipids and other phosporus-containing compounds of the mammalian nervous system, both in health and during the classical Wallerian degeneration which nerve fibres undergo following transection (see Brante, 1949; and Johnson, McNabb & Rossiter, 1950).

Demyelinating changes also occur in man after poisoning with tri-*ortho*-cresyl phosphate (TOCP), and numerous extensive outbreaks of poisoning by this compound have been described (Smith & Elvove, 1930*a*, *b*; Ter Braak, 1931; Germon, 1932; Sampson, 1938; Hunter, Perry & Evans, 1944). More recently, the biochemical effects (Earl & Thompson, 1952*a*, *b*; Earl, Thompson & Webster, 1953) accompanying the histological and clinical changes (Barnes & Denz, 1953) in experimental intoxication with this compound have been studied, and it has been found to be a potent inhibitor of the pseudo-cholinesterase present in nervous tissue.

Barnes & Denz (1953) have also shown in experimental animals that poisoning with other anticholinesterases, including di-*iso*propyl fluorophosphonate can cause very similar degenerative changes in the myelin of the spinal cord and nerve sheath, and paralysis occurring in man following intoxication by one of the 'organophosphorus' anticholinesterase insecticides has also been described by Bidstrup, Bonnell & Beckett (1953).

Since the studies of Brante (1949) and Johnson *et al.* (1950) have been confined to changes following nerve section, it seemed of interest to determine whether any change in phosphorus composition and metabolism of nervous tissue was brought about by these toxic agents. Furthermore, no report seems yet to have been made on the distribution and metabolism of phosphorus compounds in the nervous system of birds, although the hen has been used by several groups of workers as being the most suitable species in which to study, both *in vitro* and *in vivo*, the biochemical, histological and clinical effects produced by anti-cholinesterases in nervous tissue (Earl & Thompson, 1952*a*, *b*; Earl, *et al.* 1953; Barnes & Denz, 1953).

The classical use of both the pigeon and the hen for the study of thiamine deficiency, which also causes demyelination not unlike that following TOCP intoxication (Swank, 1940; Swank & Prados, 1942), provides yet another reason for obtaining biochemical data of this kind in an avian species.

A report is given here of the amount of phosphorus present in three fractions of spinal cord and sciatic nerve, namely, the acid-soluble fraction (acid-soluble P) the phospholipid fraction (lipid P) and the residue remaining after removal of acid-soluble compounds and phospholipids (residual P), and of the rate of incorporation of ^{32}P into these, *in vivo*, in normal chickens, and in chickens dosed with TOCP at varying intervals before administration of the isotope.

EXPERIMENTAL

Pairs of chickens of mixed breed, weighing between 1·5 and 3·5 kg. were used. Pure TOCP (Geigy Pharmaceuticals Ltd.), 1 ml./kg. body wt., was given to one of each pair by stomach tube, either 4 or 14 days before administration to both of ^{32}P* (100 μc as $H_3{}^{32}PO_4$ in 2 ml. 0·06 M phosphate buffer, pH 7·3) by injection into the wing vein.

Lowering of the plasma cholinesterase level 1–3 days after dosage with TOCP, as compared with that immediately before dosing, was taken as an adequate index of absorption. Cholinesterase activity was measured manometrically (Ammon, 1933), using butyrylcholine perchlorate as substrate.

The hens were kept in separate cages on a stock diet until killed by decapitation. Blood was collected from the cut end of the carotid artery into a bottle containing heparin as anticoagulant. Spinal cord, between the atlas and mid-thoracic region, was removed and the dura mater stripped off, leaving an almost blood-free preparation. In order to obtain a standard length, the main trunks of the sciatic nerves were dissected out between the knee joint and the vertebral foramina, and cleaned of connective tissue and fat. All tissues were weighed immediately after removal.

The technique for separation of the three fractions of phosphorus compounds was essentially that of Schneider (1945). A sample of spinal cord was dispersed in 11 times its weight of glass-distilled water in a $\frac{5}{8}$ in. test tube, into which was fitted a glass rod with a bulb on the end, of such size that only a very narrow clearance was left between it and the walls of the tube (Lloyd, personal communication). On moving the rod rapidly up and down, without allowing the bulb to break the liquid surface, a uniform dispersion was quickly produced. 10 ml. of this was transferred by bulb pipette to a 20 ml. test tube graduated at 15 ml., and to this was added 5 ml. of 30% (w/v) trichloroacetic acid (Hopkin and Williams, A.R.).

The paired, weighed nerves were finely minced with scissors and transferred to a $\frac{5}{8}$ in. tube with approximately 10 ml. 10% (w/v) trichloroacetic acid and the bulb-plunger used as for cord. The resulting suspension was quantitatively transferred to a test tube and made up to exactly 15 ml. with 10% trichloroacetic acid. To enable the separation of the three fractions of phosphorus compounds to be carried out without transfer of the residue after each stage, the test tubes were fitted with ground-glass joints so that a condenser or an extension tube could be attached as required (see below).

All tubes were closed with turn-over rubber caps and centrifuged. The supernatant liquid (15 ml.) contained the whole acid-soluble phosphorus fraction of the tissue taken. The residues were washed once with 10 ml. 10% trichloroacetic acid, and twice with 10 ml. water, being centrifuged each time and the washings discarded.

A condenser was fitted to the tube and lipid P extracted with three successive 5 ml. lots of a mixture of 3 vol. ethanol and 1 vol. ether, and each lot being refluxed for 5 min. in a boiling-water bath. The combined extracts, removed with a Pasteur pipette after centrifuging, were made up to 15 ml. with the solvent mixture.

The residue was digested in 1 ml. H_2SO_4 (Hopkin and Williams, MAR), and approximately 3 ml. of 100 vol. H_2O_2 (Hopkin and Williams, MAR) were added drop by drop until the contents of the tube became clear. The digest was made up to 15 ml. with water.

Acid-soluble phosphorus of plasma was analysed after precipitating proteins with a final concentration of 10% trichloroacetic acid.

Phosphorus analyses were carried out in duplicate on each fraction by a method based on that of Fiske & Subbarow (1925), on portions digested in 60% $HClO_4$ (w/v) (Hopkin and Williams, A.R.). Appropriate reagent blanks were prepared. Ascorbic acid (Hopkin and Williams, BP) in a final concentration of 0·1% was used as reducing agent, and the colours compared with standards.

Radioactivity counts were done on 10 ml. of each of the three fractions of nervous tissues and on the plasma extract, in a type M 6 liquid β Counter, and recorded for a standard time on a conventional type of scaler and timing unit. Corrections were applied in each experiment for background counts and for radioactive decay occurring during the experimental period.

* Supplied by A.E.R.E., Harwell.

RESULTS

Table 1 gives the means of the phosphorus analyses on the three fractions obtained from spinal cord and sciatic nerves of normal chickens. Means of the corresponding values for the birds 4 and 14 days after poisoning did not differ significantly from these. Since the extraction procedure was carried out in one test tube without transfer, the only point at which phosphorus might have been lost was in the washing of the residue left after extraction of acid-soluble P, and as a check on this, determinations of total P were made on samples of the original spinal-cord dispersion. The mean result obtained was 517 (S.E. ± 6·43) μg./100 mg., which does not differ significantly from the calculated total given in Table 1. The mean of the differences between the calculated and determined totals for the spinal cord of each normal bird was only 7·4 (S.E. ± 6·9) μg./100 mg.

The mean weight of the standard lengths of sciatic nerves for the birds 4 and 14 days after poisoning did not differ significantly from that of the normal nerves.

Table 2 shows the rates of uptake of ^{32}P by the three fractions expressed as relative specific activities (RSA) × 10^3, i.e.

$$\frac{\text{specific activity of acid-soluble P of tissue}}{\text{specific activity of acid-soluble P of plasma}} \times 10^3,$$

which gives an estimate of the rate of passage of ^{32}P across the blood-brain barrier, and

$$\frac{\text{specific activity of lipid P of tissue}}{\text{specific activity of acid-soluble P of tissue}} \times 10^3,$$

and

$$\frac{\text{specific activity of residual P of tissue}}{\text{specific activity of acid-soluble P of tissue}} \times 10^3$$

which give estimates of the rates of incorporation of the isotope into phospholipid and residual fractions, on the assumption that the phosphorus-containing precursor of the compounds in these fractions is a component of the acid-soluble fraction. Fig. 1 shows

Table 1. *Phosphorus distribution in chicken nervous tissue and blood plasma*

Mean ±S.E. of mean. Expressed as μg./100 mg. wet wt. for tissue, and mg./100 ml. for plasma.

	Acid-soluble phosphorus	Phospholipid phosphorus	Residual phosphorus	Total phosphorus (by addition)	No. of birds
Plasma	4·0±0·56	—	—	—	13
Cord	71·4±3·45	369±7·2	68·8±1·88	509	13
Nerve	46·0±2·98	254±8·2	39·4±2·91	339	10

Table 2. *Incorporation of ^{32}P into chicken nervous tissue*

Interval between TOCP dosage and ^{32}P injection (days)	No. of birds	Interval between ^{32}P injection and killing (hr.)	Mean 10^3 RSA* ±S.E. of mean: Acid-soluble P	Phospholipid P	Residual P
Cords					
Normal control	4	4	30·9±6·6	21·7±3·6	181±17·5
4	2	4	26·2	17·3	148
14	2	4	23·7	21·2	189
Normal control	5	24	105±9·0	75·5±5·3	410±17·0
4	3	24	116·3	67·3	396
14	2	24	109·2	65·5	370·5
Normal control	4	72	256±24·5	125±12·1	549±73·7
4	2	72	223	112	504
14	2	72	263·5	103·7	374
Nerves					
Normal control	4	4	132±32·7	14·1±0·5	243±13·2
4	2	4	164	11·8	213
14	2	4	178	18·8	209
Normal control	4	24	382±80·6	45·7±2·5	307±31·0
4	2	24	500	44·9	326
14	2	24	486	52·6	334
Normal control	2	72	634	126	396
14	2	72	704	121·8	398

* Relative specific activity (RSA) for acid-soluble P of tissue $= \frac{\text{specific activity of acid-soluble P of tissue}}{\text{specific activity of acid-soluble P of plasma}}$, for phospholipid P $= \frac{\text{specific activity of lipid P of tissue}}{\text{specific activity of acid-soluble P of tissue}}$, and for residual P $= \frac{\text{specific activity of residual P of tissue}}{\text{specific activity of acid-soluble P of tissue}}$.

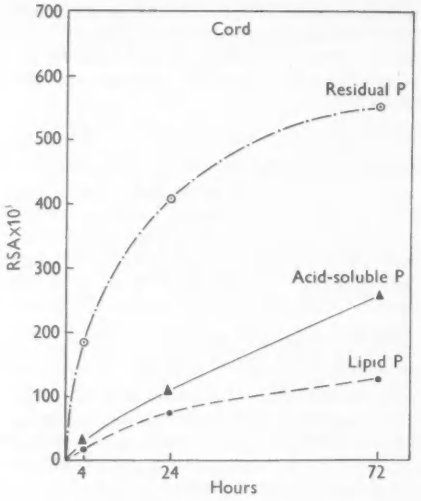

Fig. 1.

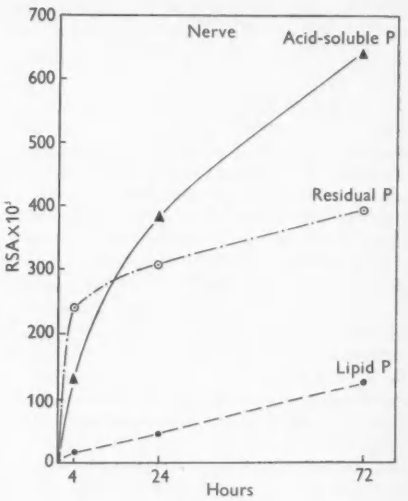

Fig. 2.

Figs. 1, 2. Incorporation of ^{32}P into phosphorus compounds of chicken nervous tissue. Acid-soluble P, ▲—▲; phospholipid P, ●---●; residual P, ⊙-·-·-⊙. Ordinate = relative specific activity (RSA), i.e.

$$\text{for tissue acid-soluble P: } \frac{\text{specific activity of acid-soluble P of tissue}}{\text{specific activity of acid-soluble P of plasma}} \times 10^3,$$

$$\text{for tissue lipid P: } \frac{\text{specific activity of lipid P of tissue}}{\text{specific activity of acid-soluble P of tissue}} \times 10^3,$$

$$\text{and for tissue residual P: } \frac{\text{specific activity of residual P of tissue}}{\text{specific activity of acid-soluble P of tissue}} \times 10^3,$$

Abscissae = time in hr. between administration of ^{32}P and killing.

the time course of the incorporation of ^{32}P into the three fractions of spinal cord, and Fig. 2 the corresponding curve for nerve.

The incorporation of ^{32}P continues to increase, but at a declining rate, during the whole of the experimental period in all three fractions of both tissues. The RSA of the residual P increases faster initially than does that of lipid P (Figs. 1 and 2).

There is no evidence of inhibition of uptake in the poisoned birds in either cord or nerve. The RSA of the residual P of spinal cord from birds poisoned 14 days before administration of ^{32}P, and killed 72 hr. after, is some 30% below the corresponding control value; but in view of the large S.E. of the latter figure this difference cannot be regarded as significant.

DISCUSSION

Composition. No attempt has been made in this work to define exactly the constitution of the three fractions of phosphorus compounds extracted by Schneider's (1945) method. In particular the residual fraction contains a variety of different types of compound. Logan, Mannell & Rossiter (1952*a*, *b*), working with mammalian white matter and peripheral nerve, found that the residual fraction contained a considerable amount of phosphorus in excess of that accounted for by nucleic acids and phosphoprotein. They suggested that this may be present as 'inositide phosphorus', which may be identical with the inositol-containing, trypsin-resistant lipoprotein complex isolated from white matter by Folch & Le Baron (1951).

The phosphorus contents of the fractions obtained from chicken nervous tissue differ from those reported for similar fractions obtained from mammalian species (Bodian & Dziewiatkowski, 1950; Samuels, Boyarsky, Gerard, Libet & Brust, 1951; Logan *et al.* 1952*a*; Logan, 1952). Whether there is also a qualitative difference in the composition of the fractions, when obtained from different species, cannot be stated without a more detailed analysis.

Metabolism. The intervals of 4 and 14 days between dosing with TOCP and administration of the isotope were chosen, because the only biochemical abnormalities so far detected in nervous tissue in intoxication with this compound are a profound inhibition of pseudo-cholinesterase, which is the predominant cholinesterase of white matter (Ord & Thompson, 1952; Thompson & Webster, 1954), and a lesser inhibition of tributyrinase (Earl *et al.* 1953). It has been shown by these authors that at 4 days after poisoning the pseudo-cholinesterase of brain and cord is maximally inhibited, while at 14 days paralysis and clearly demonstrable demyelination of the cord and nerve are present (Barnes & Denz, 1953).

Passage of ^{32}P *from blood to tissues.* The results suggest that this is slow in both cord and nerve since the relative specific activities of the acid-soluble P have not reached a maximum even after 72 hr. Bakay & Lindberg (1949), Sachs & Culbreth (1951), Rosenfeld & Beath (1952), also reported a slow passage of ^{32}P across the blood-brain barrier in mammals. From experiments showing a much faster incorporation of ^{32}P into the acid-soluble P of the central nervous system after intracisternal, as compared with intravenous, injection of the isotope, they consider that phosphate is made available to the central nervous system by a slow secretion into the cerebrospinal fluid.

Entry of ^{32}P from blood into chicken sciatic nerve is faster than into cord; the results obtained by Brierley (1952) in the rabbit were similar, the rates of entry into nerve and cord, respectively, being 11 and 1·3 times the rate of entry from blood to cerebral cortex.

Rate of incorporation of ^{32}P *into phospholipid and residual phosphorus fractions.* Many workers have reported that this is slow in brain as compared with other organs (Artom, Sarzana, Porrier, Santagelo & Segre, 1937; Changus, Chaikoff & Ruben, 1938; Chargaff, Olsen & Partington, 1940; Bodian & Dziewiatkowski, 1950; Johnson & Albert, 1953). Changus *et al.* (1938) found that fasted rats, given ^{32}P orally, showed a maximal increase in the amount of isotope present in brain phospholipid after 200 hr., while liver, kidney and intestine showed a high maximum incorporation in 10–12 hr. Fasting, for the duration of the experiment, did not appear to affect ^{32}P incorporation into brain phospholipid. The present findings, in the chicken, that the RSA of lipid P and residual P have not reached a maximum even at 72 hr. after injection of ^{32}P, appear to support the conclusion of these earlier workers, although no direct comparison with other organs was made.

Chargaff *et al.* (1940) and Dawson (1953) reported that the rates of ^{32}P incorporation differed widely between the constituents of the lipid fraction of brain, and Strickland (1952) showed that phosphoprotein incorporated the isotope faster than the other constituents of the residual fraction of cat brain. It is possible, therefore, that phosphoprotein may contribute to the steep initial rise in RSA of the residual P from chicken cord and nerve seen in Figs. 1 and 2 and Table 2.

The rates of incorporation of ^{32}P into lipid P and residual P of chicken cord and nerve are in reasonable agreement with those reported by Samuels *et al.* (1951) for guinea pig, and their finding that entry into nerve phospholipid is initially slower than into cord phospholipid is confirmed for an avian species.

Effect of TOCP in ^{32}P *incorporation.* Other workers have studied the uptake of ^{32}P into the phosphorus compounds of peripheral nerve during Wallerian degeneration following nerve section in mammals (Bodian & Dziewiatkowski, 1950; Samuels *et al.* 1951). As far as is known, no investigations of this kind have been made in animals during thiamine deficiency, poisoning with organophosphorus compounds or other experimental procedures known to produce demyelinating lesions (see Cavanagh & Thompson, 1954). In the present work it is evident from Table 2 that TOCP in a dosage sufficient to produce severe paralysis in chickens in 14 days, accompanied by histologically demonstrable demyelination in spinal cord and peripheral nerves, and by 80–90 % inhibition of pseudo-cholinesterase in these tissues, produces no significant change in ^{32}P uptake into the three fractions obtained from chicken spinal cord and sciatic nerve. Bodian & Dziewiatkowski (1950) found an increase in uptake of ^{32}P in nerve, degenerating as a result of section, in all three fractions, and suggested that this is related to the increase in Schwann cell population in this case. Nothing, however, is known as yet of the reaction of the Schwann cells in intoxication with TOCP. On the other hand, Sawyer (1946) has claimed that, following section of guinea pig sciatic nerve, the pseudo-cholinesterase of the peripheral stump is largely unaffected, and the work of Koelle (1951), Ord & Thompson (1952), and Thompson & Webster (1954) suggested the possibility that this enzyme is concerned with some aspect of myelin metabolism and that its inhibition by TOCP and certain other anti-cholinesterases may be causally related to the ensuing demyelination. The presence of an apparently normal uptake into the phospholipid and residual fractions of cord and nerve undergoing demyelination following TOCP administration does not, however, preclude the possibility that the metabolism of a component of a fraction has been interfered with, and this effect not detected by the technique used in these experiments. This would be conceivable, especially if the substance concerned

was of low metabolic activity. Taurog, Chaikoff & Perlman (1942) reported that incorporation of ^{32}P into the phospholipids of liver and kidney slices was inhibited by cyanide, carbon monoxide and anaerobiosis, while Schachner, Fries & Chaikoff (1942) found that hexose increased the incorporation into phospholipids of brain *in vitro*. Recently DeLuca, Rossiter & Strickland (1953) have shown that incorporation of the isotope into pentosenucleic acid of cat brain is dependent on an active phosphorylating mechanism. Incorporation is depressed by anaerobiosis and by inhibitors which suppress oxygen consumption, and is supported by pyruvate and lactate (Findlay, Rossiter & Strickland, 1953). The finding, therefore, of Earl *et al.* (1953), that TOCP used in the same dosage as in the experiments described in this paper had no effect on the pyruvate oxidase system of chicken spinal cord and brain, is consistent with the absence of effect on ^{32}P incorporation into lipid P and residual P.

SUMMARY

1. The phosphorus compounds in spinal cord and sciatic nerve of the chicken have been investigated, and the effect of a demyelinating anti-cholinesterase (tri-*ortho*-cresyl phosphate, TOCP) studied.

2. TOCP in dosage sufficient to cause paralysis, demonstrable demyelination and 80–90% inhibition of pseudo-cholinesterase of cord and nerve in 14 days, does not affect the amount of phosphorus in the acid-soluble, phospholipid, and residual fractions.

3. The rates of incorporation of ^{32}P into the three fractions have been measured, and a comparison made with available data on mammals. The rates appear to be slow in both tissues, and although ^{32}P enters the acid-soluble phosphorus fraction of nerve 4–5 times as fast as spinal cord, the rates of incorporation into the phospholipid and residual fractions are similar in both tissues.

4. TOCP, in the dosage used, does not appear to affect the rates of incorporation into the three fractions. The significance of this finding is discussed.

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The β-Glucuronidase of the Roman Snail (*Helix pomatia*)

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β-Glucuronidase has been found in the crop fluid of locusts (Robinson, Smith & Williams, 1953) and in the digestive gland of certain marine molluscs (Dodgson, Lewis & Spencer, 1953). The enzyme has also been reported in the digestive gland of *Helix laeda* Gould by Utusi, Huzi, Matumoto & Nagaoka (1949), but its properties were not given in detail. The enzyme used by these authors was prepared from a 'stock porridge' 3 years old.

The work reported in the present paper shows that the digestive gland of the Roman snail possesses a high β-glucuronidase activity; the other species of snails examined showed less activity. The Roman snail is a convenient object for study because of its large size, and the digestive gland, which weighs about 1·5 g., can be rapidly and easily dissected. As far as present observations go, only the digestive gland and the crop contain appreciable amounts of the enzyme. This work has been carried out in conjunction with histochemical studies on the digestive gland (Billett & McGee-Russell, unpublished observations), in an attempt to establish the localization of the enzyme.

Materials

Animals. Adult snails weighing about 40 g. were used. The snails had been kept in captivity for several months; under these conditions their food consisted mainly of cabbage. The snails were originally obtained from a colony which lives on the Cotswold Hills in the vicinity of Cheltenham.

Digestive gland. After the snail had been killed by decapitation, the digestive gland was carefully dissected out of the visceral mass. Care was taken to ensure that the gland was not contaminated with intestine and ovo-testis which are closely apposed to it.

Crop fluid. A ligature was tied round the crop behind the buccal mass. The crop was severed in front of the ligature and dissected out back towards the digestive gland. Another ligature was tied near the gland and the crop severed behind this. The crop with its contained fluid was removed to a watch glass. Puncture of the isolated crop gave 1–2 ml. of fluid. The fluid varied in appearance, but was usually a brown, rather viscous liquid. Sometimes the fluid contained undigested pieces of food; these were removed by centrifuging. Microscopic examination of the fluid failed to reveal the presence of micro-organisms. The fluid was diluted 100-fold with distilled water and assayed directly for glucuronidase.

Analytical methods

Robinson *et al.* (1953) used quinolyl-8-glucuronide as the substrate in their method for assaying the β-glucuronidase activity of locust-crop fluid, and the amount of 8-hydroxyquinoline liberated was estimated colorimetrically after coupling with diazotized di-*o*-anisidine. In the present work the same substrate was used but the 8-hydroxyquinoline formed was coupled with diazotized sulphanilic acid for purpose of estimation.

Quinolyl-8-glucuronide. This was prepared according to the method of Robinson *et al.* (1953).

Standardization of method. A standard solution of 8-hydroxyquinoline was prepared by dissolving a known weight in water made slightly alkaline with NaOH. Samples (5 ml.) of standard solution, containing 100–500 μg. of 8-hydroxyquinoline, were measured out and to each was added 1 ml. of freshly prepared diazotized sulphanilic acid in the form of Ehrlich's diazo reagent (Hawk, Oser & Summerson, 1947), followed by 2 ml. of 5N-NaOH. The diazotization was carried out at approximately 5°. After addition of the alkali the mixtures were diluted to 100 ml. with water and kept for 30 min. at room temperature to allow maximum colour development (the colour was stable for at least 24 hr. if kept in the dark). The extinction coefficients were measured at 465 mμ. in a Unicam SP. 350 Spectrophotometer (Unicam Instruments (Cambridge) Ltd.). There was a linear relationship between the extinction coefficient and amount of 8-hydroxyquinoline.

Measurement of enzyme activity. The solutions to be assayed were warmed to 25° and 1 ml. samples mixed with 1 ml. of 0·005M quinolyl-8-glucuronide in M acetate buffer, pH 4·2, and 1 ml. water. Control tubes were set up in which 1 ml. 0·001M potassium hydrogen saccharate (prepared according to Levvy, 1952) replaced the water; the saccharate solution completely inhibited the enzyme. The mixtures were incubated at 25°. After a suitable time interval, which varied between 10 min. and 2 hr., the tubes were plunged into ice-water and 1 ml. 20% (w/v) trichloroacetic acid was added to each. The mixtures were centrifuged at 1000 *g* for 10 min. and the supernatants decanted off. The residues were suspended in 3 ml. distilled water and centrifuged as above. The 8-hydroxyquinoline was estimated in the combined supernatants and washings. The colours produced required diluting twice to tenfold before a suitable reading could be obtained on the spectrophotometer. Negligible amounts of colour were produced in the controls.

Preparation of the digestive-gland extract. After being weighed, the gland was homogenized in distilled water using an all-glass homogenizer (Potter & Elvehjem, 1936). The preparation so obtained was diluted to a 1% (w/v) suspension of the tissue and sufficient toluene was added to

saturate the mixture, which was placed in a refrigerator overnight (12–16 hr.) and then centrifuged at about 1000 *g* for 10 min. After the supernatant had been decanted off, the residue was suspended in the original volume of distilled water and centrifuged under the same conditions as before. The combined extracts appeared to contain all the enzyme. The residue showed no appreciable activity. Most of the enzyme appears to be extracted within 2 hr. of homogenizing. Extractions carried out for longer than 24 hr. showed a diminished activity.

No reaction was apparent in the absence of substrate. A slight hydrolysis of the substrate occurred when it was incubated under the conditions of the assay in the absence of the enzyme preparation. Glucuronic acid in amounts equivalent to those formed by the enzymic hydrolysis of the substrate gave no colour with the diazo reagent. Complete recovery of added 8-hydroxyquinoline was obtained under the conditions of the assay.

After it had been established that the reaction rate was approximately linear for the first hour a series of determinations were made using an incubation period of 40 min.

RESULTS

Optimum substrate concentration. The activity of the digestive-gland extracts was measured at substrate concentrations ranging from 4×10^{-4} to 3×10^{-3} M, at pH 4·2. The result is shown in Fig. 1 and indicates an optimum substrate concentration in the region of 0·002 to 0·003 M. The solubility of quinolyl-8-glucuronide in M acetate buffer, pH 4·2, is between 0·005 and 0·01 M at about 18°. Because of this low solubility a slightly sub-optimum substrate concentration was used in the main determinations.

Optimum pH. Determinations were carried out as already described except that the pH of the acetate buffer was varied from 3·7 to 5·5. The pH/activity curve is shown in Fig. 2. The optimum pH appears to be in the region of 4·2.

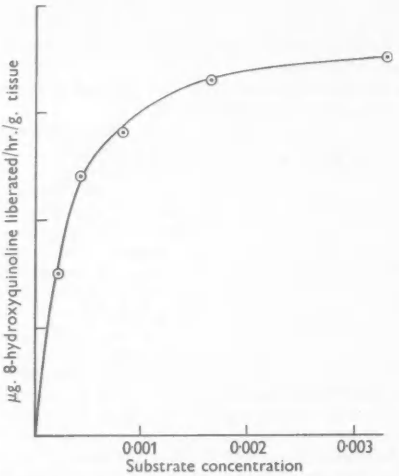

Fig. 1. Substrate concentration/activity curve for β-glucuronidase of *H. pomatia*. The substrate was quinolyl-8-glucuronide in 0·33 M acetate buffer, pH 4·2; temp. 25°.

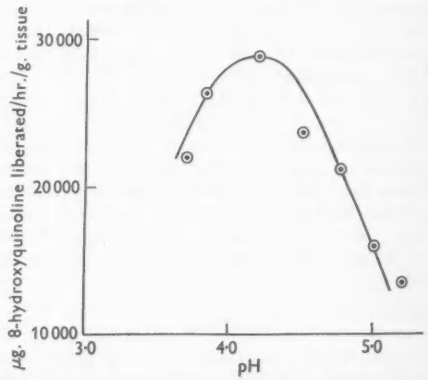

Fig. 2. pH/activity curve for *H. pomatia* β-glucuronidase. The substrate was 0·0017 M quinolyl-8-glucuronide in 0·33 M acetate buffer; temp. 25°.

Table 1. *β-Glucuronidase activity of mollusc extracts at* 25°

The substrate was 0·0018 M quinolyl-8-glucuronide in M acetate buffer, pH 4·2. The activity is expressed in terms of μg. of 8-hydroxyquinoline/hr./g. or ml.

Species	Tissue	Activity		No. of determinations
		Mean	Range	
Helix pomatia	Digestive gland	30 900	18 900–45 000	14
	Crop fluid	12 200	5 400–16 500	6
	Kidney	400	0–900	4
	Albumen gland	1 600	1 170–2 100	4
	Salivary gland	700	0–1 200	4
	Mantle ridge	50	0–200	4
	Foot	400	0–1 200	4
	Ovo-testis	2 500	700–7 200	4
H. aspersa	Digestive gland	13 500	8 600–17 000	6
Limnea stagnalis	Digestive gland	500	0–1 800	6
Anodonta cygnea	Digestive gland	4 800	4 400–5 600	6
Planorbis corneus	Digestive gland	4 300	2 800–5 400	6

Activity of the digestive-gland extracts and crop fluid. After optimum conditions of substrate concentration and pH had been established, a series of consecutive determinations were made on the enzyme preparations. Each preparation was obtained from a separate snail. The results are given in Table 1. The activities are expressed as μg. of 8-hydroxyquinoline liberated/g. of tissue/hr. in the case of the digestive gland and as μg. of 8-hydroxyquinoline/ml./hr. in the case of the crop fluid.

Distribution of β-glucuronidase in organs of H. pomatia *and other molluscs.* Included in Table 1 are the results of determinations on a number of organs of *H. pomatia* besides the digestive gland. The activities of the digestive glands of the garden snail, two fresh-water gastropods and the swan mussel were also determined in the same way and under the same conditions as those described for the digestive gland of *H. pomatia*.

DISCUSSION

The results show that the digestive glands of *H. pomatia* and *H. aspersa* contain appreciable amounts of β-glucuronidase. The glucuronidase activity of these terrestrial species is significantly higher than that of certain fresh-water species. In the Roman snail, high enzyme activity is confined to the digestive gland. The albumen gland appears to possess a slight activity. No significance can be attached to the activity associated with the ovotestis because it is difficult to dissect this organ free of the investing digestive gland.

The digestive gland of the Roman snail appears to be one of the richest sources of glucuronidase so far discovered. The activity is comparable with that of locust-crop fluid (Robinson *et al.* 1953) and of the digestive gland of marine molluscs (Dodgson *et al.* 1953); it is probably greater. A direct comparison is not possible because of the different conditions of assay used by the various authors. In particular it must be noted that, in the experiments described in this paper, incubation was at 25°, which is nearer to that normally obtaining in the animal than is 37°; at the latter temperature the activity of the extracts is more than doubled.

The pH optimum of 4·2 found for *H. pomatia* glucuronidase is to be compared with the value 4·5, found by Robinson *et al.* (1953) for locust-crop fluid using the same substrate. Dodgson *et al.* (1953) found an optimum pH of 4·0 for the glucuronidase of marine molluscs using *p*-chlorophenylglucuronide as substrate. These pH data suggest a fairly close relationship for the enzymes from invertebrate sources. The present work has not revealed a second optimum pH which appears to be a characteristic of the enzyme isolated from certain vertebrate tissues (Mills, 1948; Kerr, Campbell & Levvy, 1949). Utusi *et al.* (1949) investigated the action of glucuronidase from the digestive gland of *H. laeda* on 0·025 M β-menthol glucuronide in citrate buffer, pH 4·5. About 30 % of the substrate was hydrolysed after incubating for 1 hr. at 37°. Optimum conditions do not appear to have been determined.

The function of the enzyme in molluscs is probably similar to that in locusts and in the micro-organisms which exist in the gut of ruminants (Marsh, Alexander & Levvy, 1952). As suggested by the various authors concerned, the enzyme probably acts on a substance containing a β-glucuronide link which normally exists in the food of these herbivorous animals. This substance may be hemicellulose (Marsh *et al.* 1952). The glucuronidase of the snail may be considered to be a component of 'cytase', a mixture of enzymes concerned with the breakdown of cellulose and related substances (Faberge, 1945; Holden, Pirie & Tracy, 1950; Holden & Tracy, 1950).

Histochemical investigations (Billett & McGee-Russell, unpublished observations) indicate that the enzyme is localized in particular cells in the digestive gland of the snail. These investigations suggest that the tissue itself, and not micro-organisms which may reside in the crop and digestive gland, is responsible for the production of the enzyme which is found in the crop fluid.

SUMMARY

1. The digestive gland and the crop of the Roman snail, *Helix pomatia*, appear to contain large amounts of β-glucuronidase. This enzyme has an optimum pH of 4·2 when acting on quinolyl-8-glucuronide as a substrate.

2. The 8-hydroxyquinoline formed by the enzymic hydrolysis of the substrate was estimated by coupling it with diazotized sulphanilic acid. The concentration of the azo dye produced was measured spectrophotometrically.

3. The β-glucuronidase of the Roman snail is related to, and probably identical with, the enzyme known to exist in the digestive gland of certain marine molluscs and the crop fluid of locusts.

4. The digestive glands of *Limnea stagnalis*, *Planorbis corneus* and *Anodonta cygnea* contain only small amounts of glucuronidase compared with *Helix pomatia* and *H. aspersa*.

I wish to thank Dr J. R. Baker for his interest in my work and the encouragement he has given me. Prof. A. C. Hardy kindly provided the laboratory facilities. I am especially grateful to S. M. McGee-Russell for his help in dissecting the snails and for much useful discussion during the course of the work. I am indebted to Dr J. N. Smith for a sample of quinolyl-8-glucuronide and for some information on its method of preparation before this had been published. The Medical Research Council provided a personal grant.

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β-Amylolysis: Union of Enzyme and Substrate

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It is well known that β-amylase liberates maltose from starch and its components in the β form (Kuhn, 1925; Freeman & Hopkins, 1936). This was established by observing the mutarotation of the fission products and establishing that it was the maltose itself which was undergoing this change. In one type of experiment the optical rotation of the β-amylase/starch reaction mixture is read continuously during the reaction and samples are withdrawn from time to time and treated with alkali to stop enzyme action and hasten mutarotation. The rotations of the former series are lower than the corresponding ones of the latter (corrected for dilution) because β-maltose ($[\alpha]_D + 118°$) has yet to change into αβ-maltose ($[\alpha]_D + 137°$).

In repeating some experiments of this kind and taking direct readings of optical rotation of the reaction mixture very soon after mixing the enzyme and substrate it was observed that the rotation was higher, not lower, than that of the corresponding mutarotated solution. After the reaction had proceeded further this position was reversed and subsequent readings conformed to those previously reported by the above authors. The phenomenon was confirmed with starch, amylose and a short-chain amylose dextrin and was considered to be well outside the range of experimental error. The simplest explanation is that an enzyme-substrate compound is formed, the optical rotation of which (dextrorotatory) is greater than that of the substrate.

MATERIALS AND METHODS

Substrates. Soluble starch was prepared from potato starch by the method of Hanes & Cattle (1938), $[\alpha]_D^{20} + 198°$ in water; amylose from potato starch by precipitation with thymol followed by repeated recrystallizations with butanol as described by Hopkins & Jelinek (1948). Achroic amylose dextrin was prepared by hydrolysing amylose with bacterial α-amylase (prepared from *B. subtilis*) to the achroic stage and its separation from the lower-molecular hydrolytic products in a charcoal-Celite column as described by Bailey, Whelan & Peat (1950). The lower fractions eluted by ethanol up to 25% (v/v) were separated and the remainder eluted with ethanol (30%, v/v, and higher) but not further fractionated, $[\alpha]_D^{20} + 186°$ in water.

Enzymes. We are indebted to the Wallerstein Laboratories, New York, for a preparation of β-amylase of high purity from ungerminated barley, and to Dr A. K. Balls for crystalline sweet potato β-amylase (4 times recrystallized).

Reactions were performed as follows. The substrate and enzyme were prepared in solution, and after standing for a period of 1 hr. at the temperature at which the reaction was to be carried out, the optical rotation of each was measured in the polarimeter at that temperature. Our soluble starch fell in $[\alpha]_D$ by about 0·6 angular degree for each degree (° c) rise in temperature. In the case of amylose special precautions were necessary, such as reading immediately after filling the polarimeter tube (see Results). The reaction mixtures contained no added buffers, the pH being about 6.

On mixing suitable volumes of amylase (5 ml.) and substrate (100 ml.), after a short vigorous shaking a portion was transferred to a jacketed polarimeter tube and the first reading obtained as quickly as possible, and later readings at intervals. These are represented graphically in Figs. 1–3 and are described there as 'continuous' curves. The remainder of the reaction mixture was kept in a flask in a water bath at the same temperature. Samples (10 ml.) were withdrawn at intervals and rapidly run into 1 ml. of 0·2 N-NaOH. After about 30 min. the optical rotation was measured at the reaction temperature. The results (corrected for dilution) are shown in the figures as 'withdrawals' curves. Such small fall in α_D of starch or other substrate or maltose as was caused by the alkali (after correcting for dilution) was allowed for, control experiments having been performed. Portions of the final reaction mixture, after amylolysis had ceased, treated with equal volumes of NaOH and

water in parallel withdrawals, gave with the alkali a small fall in α_D, about 1–2% of its value. Similar results were obtained if the reaction was checked with 0·2N ammonia instead of NaOH.

In the cases of amylose and the achroic dextrin, the progress of the reaction was also followed by determining reducing power by the method of Blom & Rosted (1947). From these data the values of α_D for the mutarotated products were calculated on the assumption of a linear relationship between increase of reducing power and fall in α_D over the range of the experiment. This has received subsequent justification by the observations reported by Whelan, Bailey & Roberts (1953) that the Freudenberg relationship holds for these unbranched short-chain dextrins from maltose upwards.

The curves in Figs. 1–3 described there as 'β-maltose' curves were obtained in the following way by calculation from the data of the 'withdrawals' curves. Our starch and amylose had $[\alpha]_D^{20}+198°$. Taking that of αβ-maltose as +137° and of β-maltose as +118° and allowing for the increase in weight resulting from hydrolysis, it was calculated that the fall in α_D in producing β-maltose would be 1·37 times as great as that for the formation of an equal quantity of αβ-maltose. (Actually the fall in α_D during β-amylolysis of starch is partly due to changes in $[\alpha]_D$ of the residue, but the correction arising from this is small and does not affect the conclusion.)

Both the 'withdrawals' and the 'β-maltose' curves start from the initial value of α_D calculated from the values of α_D of substrate and amylase (the amylase contributing less than 1% of this initial rotation). The 'continuous' curve was extrapolated to the zero axis.

RESULTS

Soluble starch, alone or previously partly hydrolysed by β-amylase, when treated with NaOH, as described, fell in value of α_D by about 1·5% of its original value. Enzyme added to starch so pretreated was inactivated, since the value of α_D of the mixture was and remained that which was calculated from those of the enzyme and alkali-treated starch.

Soluble starch undergoing hydrolysis by β-amylase exhibited values of α_D as shown in Fig. 1, amylose and its achroic dextrin as in Figs. 2 and 3 respectively. When the reading could be made soon enough, the value exceeded that calculated for the initial mixture. In all cases the early readings were

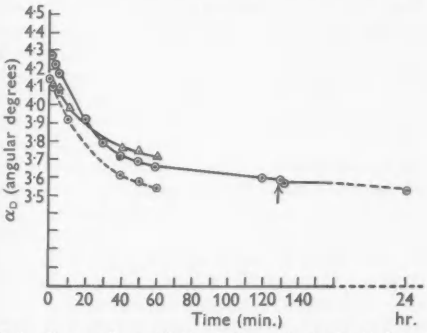

Fig. 1. Change in optical rotation of starch during β-amylolysis. Soluble starch (2·18 g. in 100 ml. solution) was mixed with Wallerstein β-amylase (2·5 mg. in 5 ml. solution) at 16°. α_D was read in a 1 dm. tube. ⊙—⊙, 'Continuous' curve, readings of reaction mixture; △—△, 'withdrawals' curve, readings after addition of alkali (corrected for dilution, see text); ⊙- - -⊙, calculated 'β-maltose' curve (see text). After 130 min. 2·5 mg. more β-amylase were added.

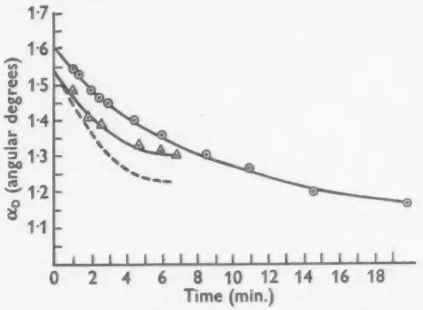

Fig. 2. Change in optical rotation of amylose during β-amylolysis. Amylose solution (0·425 g; 100 ml.) was mixed with Wallerstein β-amylase (100 mg. in 10 ml. solution) at 16°. α_D was read in a 2 dm. tube. Symbols as in Fig. 1. The reading at 6 min. on the 'withdrawals' curve was calculated from a reducing-power determination.

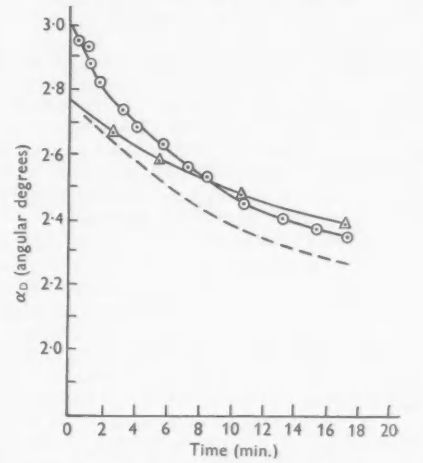

Fig. 3. Change in optical rotation of achroic dextrin during β-amylolysis. Achroic dextrin (0·765 g. in 100 ml. solution) was mixed with Wallerstein β-amylase (25 mg. in 5 ml. solution) at 19°. α_D was read in a 2 dm. tube. Symbols as in Fig. 1; α_D in 'withdrawals' curve calculated from reducing power.

Table 1. *Optical rotations of reaction mixtures at the start and end of β-amylolysis*

Readings after addition of NaOH were corrected for dilution and for the small effect of the NaOH on the carbohydrate.

			Optical rotation, α_D, in 2 dm. tube (°)				
			At start		At end (or after slowing down)		
Substrate	β-Amylase	Temp. (°)	Without NaOH (extrapolated from 'continuous' curve)	Calculated from α_D of constituents	Without NaOH	With NaOH	R_m*
Starch	Wallerstein	16	Approx. 4·5	4·32	3·62	3·61	61
	Wallerstein	8	7·40	7·08	—	—	—
	Crystalline	19	3·68	3·55	3·05	3·02	56
	Crystalline	16	4·82	4·64	4·45†	4·27†	30
					4·17‡	4·06‡	47
Amylose	Crystalline	16	—	2·04	1·65	1·64	74
	Crystalline	16	—	1·72	1·37	1·39	72
	Crystalline	16	Approx. 1·55	1·50	1·16	1·17	82
	Crystalline	17	Approx. 1·70	1·63	1·28	1·28	80

* Reducing power as percentage of theoretically obtainable maltose.
† Readings taken after the initially fast reaction had slowed down markedly.
‡ Readings taken when the reaction had ceased.

Table 2. *Changes in optical rotation of starch solution during α-amylolysis*

The digest contained Lintner soluble starch (2 g.) and malt α-amylase (≡100 g. malt) in 0·015 N acetate (125 ml.; pH 4·6) at 20°.

Time (sec.)	α_D in 1 dm. tube (°)	R_m*
0	2·94	0
60	3·04	—
120	3·09	—
130	3·06	25·8
24 hr.	2·18	83·6

* For definition, see Table 1.

higher than those of corresponding withdrawals corrected for dilution and for the small effect of the alkali. The curve of the former, however, ultimately intersected that of the alkali-treated withdrawals. The latter, redrawn to represent the corresponding rotation if the maltose present had been solely in the β-form, remained more or less uniformly below the curve of the untreated reaction mixture.

The general patterns of the curves were similar in all the experiments, i.e. for different substrates and for reactions at different rates. Of course the rate must be fast enough to show the phenomenon. A relatively slow reaction gave time to take the necessary readings and to show the rise brought about by mixing enzyme and substrate, before amylolysis had caused sufficient fall to mask this rise (Fig. 1). The phenomenon was obtained at three temperatures (Table 1), with the Wallerstein enzyme, the Balls crystalline β-amylase and with barley and soya amylases prepared in these laboratories (results not given).

When amylose was used, certain difficulties ensued which were attributed to birefringence and to the rapidity with which this surface-active colloid concentrated on the walls of the polarimeter tube. This was most marked when the crystalline enzyme was used. Fig. 2 shows one successful experiment with amylose. Less surface activity was shown by amylose prepared by leaching potato starch at 63° (described by Hopkins & Jha (1950) and termed by them 'Baldwin' amylose). The difficulty was avoided by the use of the achroic amylose dextrin (Fig. 3).

With all substrates, the abnormal rotatory power had disappeared by the end of amylolysis (see Table 1). It was not possible to demonstrate the stage at which this had happened (presumably a gradual process) because mutarotation masked it.

In one or two cases in which crystalline β-amylase was used the amylolysis became quite slow at a stage appreciably before the usual limit, and the accompanying mutarotation became negligible. In such cases the readings of α_D taken before still exceeded those taken after the addition of alkali (Table 1).

In one experiment (Fig. 1) a further addition of enzyme was made after the original reaction had become very slow. No rise in α_D was observed.

No rise or change in α_D was observed in the following control experiments: (i) when active amylase was replaced by its equivalent of inactivated β-amylase preparation, (ii) when a very active preparation of yeast saccharase was mixed with soluble starch, (iii) when β-amylase was added to amylopectin β-limit dextrin (α-amylodextrin).

A similar rise with malt α-amylase is clearly seen in the results reported by Freeman & Hopkins (1936), the essential figures of which are reproduced in Table 2. A flocculum formed in the reaction mixture very soon after the observed rise in rotation and prevented further direct readings.

DISCUSSION

When β-amylase is mixed with starch or the other substrates used, including the dialysable achroic dextrin, there is an immediate rise in optical rotation, and the raised value can be observed before the fall due to amylolysis masks it. Subsequently to this, comparison of α_D of the reaction mixture with that observed in portions in which the reaction is arrested and mutarotation completed, shows that the former continues to be the higher. Later still, even after this has ceased to be the case, the value of α_D exceeds that which is to be expected on the basis that the maltose which has been liberated is in the β form. The 'continuous' curve remains above and approximately parallel to the 'β-maltose' curve until the reaction stops or mutarotation becomes appreciable. Under the conditions of experiment (pH 6, no added salts) the effect of mutarotation of β-maltose would be negligible for several minutes after the reactions were commenced. (For velocity constants of mutarotation see Freeman & Hopkins, 1936; Isbell & Pigman, 1937.)

This abnormality in optical rotation ceases when the amylolysis ceases and is not exhibited when β-amylase is added to the reaction mixture at the end of the reaction (Fig. 1), or to amylopectin β-limit dextrin, on which β-amylase does not act. Nor was it observed when starch was mixed with heat-inactivated β-amylase or with an active enzyme such as saccharase which does not function with starch. The colloidal character of starch and amylose is not responsible since the dialysable achroic amylose dextrin exhibits the phenomenon.

One is led to the conclusion that the rise in optical rotation is associated with the enzymic reaction and is due to the formation of an enzyme-substrate compound. The rise is appreciable and is equivalent to about 3–4 % of the optical rotation of the substrate. It is about equal to the rotatory power that would be lost on the fission of 6–8 % of the α-1:4-glucosidic linkages followed by change of the reducing groups so formed to the αβ equilibrium. This suggests that the enzyme unites specifically with more glucosidic groups than the two concerned in the fission, and indeed it is now considered that this must be the case. β-Amylase does not hydrolyse α- or β-maltosides with which it has been tested (Helferich & Petersen, 1935), phosphohexaose (Posternak, 1951), maltotriose only with difficulty (Bailey *et al.* 1950; Whelan *et al.* 1953) and is apparently only able to unite with and hydrolyse efficiently maltotetraose and longer chains. In passing it may be added that malt α-amylase exhibits this phenomenon of increase in rotation in such degree as to suggest union with an even greater length of chain, but this merits proper investigation with crystalline enzymes.

The conception of a multipoint contact between enzyme and substrate (Gottschalk, 1950) is probably nowhere more appropriate than in considerations of the amylases, and it is to such a union that the optical rotatory changes are mainly to be ascribed. However, there is another factor. In the cases of both α- and β-amylase the change in optical rotation is in the positive sense. As the α-glucosidic linkage which is split by these enzymes itself contributes appreciably in the positive sense to the rotatory power of the polysaccharide, the strain imposed on this linkage by the enzyme might well increase this contribution.

SUMMARY

1. On mixing β-amylase with a solution of starch, amylose or an achroic dextrin, an initial increase in optical rotation takes place.

2. During the β-amylolysis reaction, the optical rotation of the mixture remains above that calculated from the values of the enzyme, substrate and fission products.

3. The rise in optical rotation is ascribed to the formation of an enzyme-substrate compound, and is regarded as experimental evidence of such.

4. A similar rise in optical rotation has been reported when α-malt amylase is mixed with Lintner soluble starch.

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The Estimation of Serum Proteins by Electrophoresis on Filter Paper

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The publication of a method for separation of proteins by electrophoresis on filter paper (von Turba & Enenkel, 1950) has led to widespread application, and already many reports have appeared of its use in qualitative analysis.

Doubt has been cast on the quantitative applicability of the method (Slater & Kunkel, 1953), but evidence will be presented showing that results can be obtained, accurate for any component of a mixture to within 6% of the total protein present.

EXPERIMENTAL

Method

Three types of apparatus have been described; all three aim at minimizing evaporation from the paper surface during the separation by enclosing the paper in an atmosphere saturated with water vapour.

Glass plates (Cremer & Tiselius, 1950; Kunkel & Tiselius, 1951). The paper strip is clamped between two glass plates treated with silicone. This is technically the most difficult method, but the only one by which mobilities can be measured. In this method the spots or bands of protein often become rather diffuse.

The 'Gallows' method (Durrum, 1950; Flynn & de Mayo, 1951). The paper is suspended at the mid-point over a raised glass rod. This method gives satisfactory separations; care is needed to avoid the production of an artifact due to a pool of protein solution collecting between the paper and the rod over which it lies.

The box type of apparatus (Grassman, Hannig & Knedel, 1951). This is the type that has been used in the ensuing experiments (Fig. 1). The boxes are of Perspex, 9 × 5 × 2 in., a small flange is attached at each end to hold the paper taut, and the bottom of the box is lined with lint soaked in distilled water. The boxes are arranged in pairs bridging troughs containing buffer; the fluid levels in the troughs are kept equal by connexions of rubber tubing, in which are incorporated short lengths of 1 mm. glass capillary (Fig. 1). The two end troughs connect through a buffer-agar (1%) bridge to electrode vessels filled with buffer and containing carbon electrodes. These are connected to a source of e.m.f., controllable up to 250 v d.c.

If the protein is applied at the cathode end of the paper, then evaporation of buffer from the paper during the run leads to a continual flow of buffer towards the centre of the paper, which carries the protein forward from the origin. The endosmotic flow, which carries the γ-globulin behind the starting point in other methods, has a smaller effect so that all the fractions advance beyond the origin (Fig. 2); this is occasionally of advantage in excluding the presence in the sample of denatured protein which remains at the origin.

Technique

The filter paper (Whatman no. 3MM or no. 100) is cut into strips measuring 6 × 19 cm. A transverse line is drawn about 12 cm. from one end and five pencil marks are made on this line at 0·5 cm. intervals over the middle 2 cm. The paper is soaked in barbitone buffer pH 8·6, $I/2 = 0{\cdot}05$ (0·1 M sodium diethyl barbiturate, 0·02 M diethyl barbituric acid), and laid on filter paper to remove any excess. Five spots of 0·01 ml. of protein solution, total concentration not more than 1 g./100 ml., are put on each pencil mark with a pipette. The strips are placed in the boxes with the protein about 5 cm. from the cathode end, and the two free ends arranged to dip into the buffer troughs. With the apparatus illustrated six strips are run concurrently.

A current of 1·5 mA is passed through each strip for 18 hr. (approx. 100 v/strip). In albumin-containing solutions a trace of bromophenol blue may be added; this will bind to the albumin and act as a marker of the length of run achieved. After separation the strips are dried in an oven at

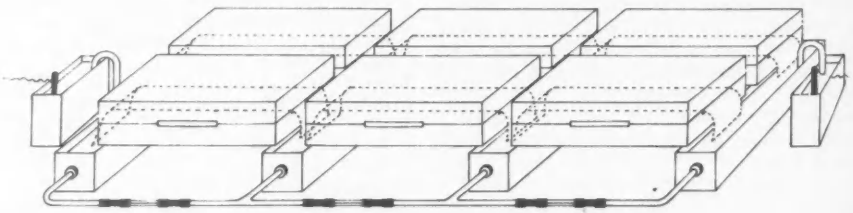

Fig. 1. Apparatus used for electrophoresis of serum on filter paper.

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100°, pinned on a frame with glass pins, fixed and stained in ethanol saturated with mercuric chloride (33 g./100 ml., w/v) containing 0·1% bromophenol blue. After 30 min. the papers are washed in 0·5% acetic acid in water until the background is clear (four washes over 40 min.), and finally dried in the oven at 100°.

Total proteins are estimated by the biuret method (Wolfson, Cohn, Calvery & Ichiba, 1948), using a serum-albumin standard. For differential determination the strips are cut up in segments of measured width, each segment incorporating one of the visible protein fractions, or the clear area between two such fractions. Blank samples of 10 mm. width are taken at the ends of the paper, over which no protein has run (Fig. 3). Each segment is eluted with 7 ml. of 2% (w/v) Na_2CO_3 in 50% methanol (v/v). Each eluate is compared with elution fluid in a Hilger Spekker Absorptiometer with a filter giving maximal transmission at 6000 Å. The appropriate blank value, in respect of the segment size, is subtracted from each eluate, together with the allowance for albumin 'tail' (see below). The content of each fraction is then worked out as a percentage of the total density, any segments from between two adjacent fractions being divided between them in proportion to their respective densities.

Preliminary experiments with 'scanning' of cleared strips in a photo-electric colorimeter confirmed the observation of Crook, Harris & Warren (1952) that densities obtained are not linearly related to the quantity of protein applied; no further investigation of this method has been pursued.

In clinical work separation of serum has been preferred to plasma, since the fibrinogen confuses the electrophoretic separation of β- and γ-globulins.

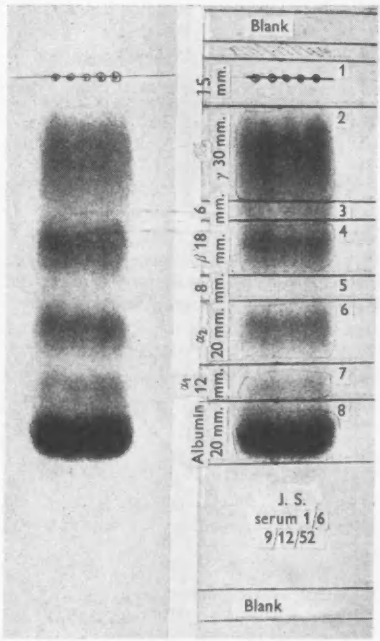

Fig. 2. Fig. 3.

Fig. 2. Separation of 0·05 ml. of serum (6·5 g. protein/100 ml.) diluted ×6 in barbitone buffer. The protein was applied in five drops of 0·01 ml. to the five spots marked on the paper; run at 1·5 mA for 16 hr.

Fig. 3. Strip shown in Fig. 2 marked out for elution.

RESULTS

Control experiments

In developing this method, rigid standardization has been necessary to obtain quantitative results. With the technique described, reproducibility and recovery is within ±6% of the total protein or density involved.

Representative experiments investigating a number of critical variables are described.

Concentration of dye and time of staining. Only bromophenol blue has been used, since this dye is readily and completely eluted from the paper. Maximum staining is obtained within 20 min. of application. There was little increase in density obtained when solutions containing 0·1, 1·0 and 2·5% dye were used for staining. Quantitative results were satisfactory with the most dilute preparation.

Washing solutions. (i) Birmingham tap water, pH of 7·0, elutes the dye steadily from the paper and there is a strong possibility of uneven washing occurring. (ii) Ethanolic solutions rapidly elute a large proportion of the dye from the protein fractions. The rate of elution is equal for albumin and γ-globulin. The method is expensive, and unsatisfactory in that maximum staining is not obtained. (iii) In 0·5% acetic acid no appreciable amount of dye was lost from the protein when samples washed 10 min., 30 min. and 24 hr. were compared. This method therefore gives maximum dye density/unit concentration of protein, and is the method of choice.

Reproducibility of staining. Absolute reproducibility of staining for a given protein concentration from day to day has not been obtained, though the proportionality of staining between fractions in a mixture of proteins remains the same. In strips run concurrently and stained together, staining is more consistently related to concentration.

Optimum concentrations of protein solution were placed on the paper, fixed and stained, and optical density plotted against protein concentration. By putting on more of a less concentrated solution of human serum albumin, estimated to be about 90% pure by U-tube electrophoresis, it was possible to obtain larger-sized spots. Fig. 4 shows the result of such an experiment; gross understaining of protein occurs if the concentration on the paper is too great (probably due to the fixed coagulum becoming impermeable to the dye). Even with spots of an area of 11 cm.² the protein concentration cannot be taken much above 0·05 ml. of a 0·8% solution.

When the protein is run electrophoretically before staining, slightly better results are obtained, and with a spot area of 10 cm.², the density is linear up to 0·05 ml. of a 1·1 g./100 ml. solution. This understaining or 'packing' is liable to occur if the concentration of any one fraction exceeds 1·0 g./100 ml.

As a result of using dilute protein solutions, the staining of small components (e.g. α_1-globulin) becomes very faint; by putting up a line of $5 \times 0{\cdot}01$ ml., even bands of separation of adequate intensity for visual examination and quantitative estimation are obtained. Applying the protein as a hair line with a paint brush is particularly liable to produce 'packing' effects, and is not therefore recommended.

Light absorption of bromophenol blue colour with the filter used was linear with concentration to an optical density of 1·8.

Elution of the dye with sodium carbonate gives complete extraction, and the colour is stable for 24 hr. The dye fades rapidly in 0·01 N sodium hydroxide.

Type of paper. With the thinner papers (Whatman nos. 1, 2 and 4) very sharp bands of separation, particularly suitable for qualitative analysis, are obtained. 'Packing' effects however occur at very low protein concentration. Whatman no. 3 MM and no. 100 have been found satisfactory for quantitative estimation.

'Tailing'. To check whether any 'tailing' of protein occurred on the paper, a number of strips were run containing albumin marked by bromophenol blue; the blue spot was cut out while still moist, and eluted from the papers in buffer. This albumin was pure in that it had already once travelled over the paper; on running again, however, it was again found to leave a 'tail'. This tail was less on Whatman no. 3 MM than on no. 31 or no. 100 papers, and amounted to an optical density of 0·001/mm. of paper passed, irrespective of the amount of protein applied; if the solution were sufficiently dilute, then all the albumin was left in the tail and no spot appeared. As a further check, a preparation of albumin (Lister transfusion) marked with bromophenol blue was run for 16 hr., the distance migrated was noted, and the current reversed until the albumin had returned half-way to the origin; staining and elution revealed that the albumin had left some protein behind on its return passage over the paper (Fig. 5). There is little doubt that other fractions leave a similar tail, but sufficiently pure preparations have not been obtained to estimate these quantitatively; allowance is made for the albumin tail by adding 0·001 to the density of albumin for every mm. of travel, and subtracting the equivalent amount from each globulin fraction. The correction is particularly important for albumin, since it may represent an increase of 5–10 % in the estimated amounts of other components.

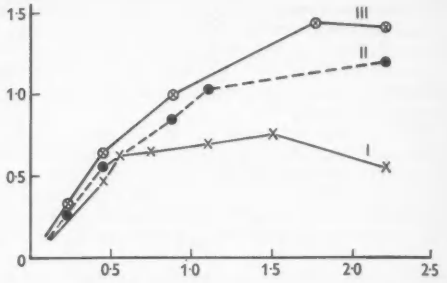

Fig. 4. Effect of spot area on staining. Ordinate, optical density; abscissa, protein g./100 ml. I, area = 4 cm.²; II, area = 7 cm.²; III, area = 11 cm.²

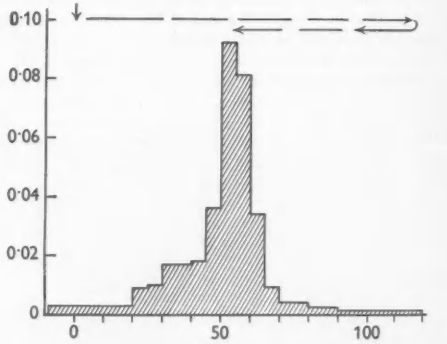

Fig. 5. Effect of reversing current on migration of albumin (90 % pure). The broken line shows the path of albumin migration. The arrow shows the starting point. Ordinate, optical density/mm. of paper. Abscissa, distance (mm.).

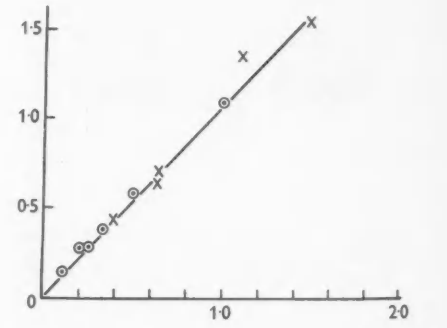

Fig. 6. Dye-binding capacity of albumin and 'cold insoluble globulin'. ×, Albumin; ⊙, cold insoluble globulin. In each case three drops of 0·005 ml. of solution were applied. Ordinate, optical density. Abscissa, g. protein/100 ml.

Table 1. *Recoveries of albumin and globulin from mixtures*

	Albumin (ml.)	Globulin (ml.)	Found (%)				Expected (%)			
			Albumin	α_2	β	γ	Albumin	α_2	β	γ
I	1·0	0	90	—	10	—	—	—	—	—
II	0·8	0·2	64·0	4·8	3·7	27·5	69·5	1·3	7·3	21·9
III	0·6	0·4	51·4	8·7	4·9	34·8	52·0	2·5	5·1	40·4
IV	0·4	0·6	32·3	4·5	5·7	57·6	37·2	3·4	3·2	56·2
V	0·2	0·8	21·2	5·5	0	73·3	24·2	4·3	1·5	70·0
VI	0	1·0	13·0	5·0	0	82·0	—	—	—	—

In each case five 0·01 ml. portions of mixture were applied and run at 1·5 mA for 16 hr. Albumin solution = 1·1 g./100 ml. Globulin solution = 1·6 g./100 ml.

Table 2. *Recovery of albumin from serum*

Case B.W. (i) Serum protein 6·3 g./100 ml. diluted × 3 (2·1 g./100 ml.), five 0·005 ml. spots. (ii) Albumin (Lister 100% electrophoretically) (1·06 g./100 ml.), five 0·005 ml. spots. (iii) Mixture 1 vol. (i) + 1 vol. (ii).

	(i) Serum (g./100 ml.)	(ii) Albumin (g./100 ml.)	(iii) Found (g./100 ml.)	(iv) Expected (g./100 ml.)	Deviation % total
Albumin	1·09	1·06	2·13	2·15	−0·5
α_1	0·12	—	0·13	0·12	+0·5
α_2	0·24	—	0·25	0·24	+0·5
β	0·19	—	0·29	0·19	+3·2
γ	0·45	—	0·35	0·45	−3·2
			3·15	3·15	

Table 3. *Recovery of fractions from mixtures of serum and urine*

	(i) Serum (g./100 ml.)	(ii) Urine (g./100 ml.)	(iii) Found (g./100 ml.)	(iv) Expected (g./100 ml.)	Deviation % total
Case K.W. (i) Serum diluted × 2 (2·1 g./100 ml.). (ii) Urine (0·84 g./100 ml.). (iii) Mixture 1 vol. (i) + 1 vol. (ii).					
Albumin	0·67	0·72	0·60	0·70	−6·8
α_1	0·09	0·05	0·06	0·07	−0·75
α_2	0·71	0	0·33	0·35	−1·4
β	0·38	0·04	0·26	0·21	+3·4
γ	0·25	0·03	0·22	0·14	+5·4
			1·47	1·47	
Case M.F. (i) Serum diluted × 1·5 (1·8 g./100 ml.). (ii) Urine (1·6 g./100 ml.). (iii) Mixture 1 vol. (i) + 1 vol. (ii).					
Albumin	0·47	0·88	0·79	0·68	+6·5
α_1	0·10	0·23	0·12	0·16	−2·5
α_2	0·54	0·15	0·31	0·35	−2·5
β	0·48	0·20	0·39	0·34	+3
γ	0·21	0·14	0·09	0·17	−4·7
			1·70	1·70	
Case J.R. (i) Serum diluted × 2 (2·2 g./100 ml.). (ii) Urine diluted × 2 (0·99 g./100 ml.). (iii) Mixture 1 vol. (i) + 1 vol. (ii).					
Albumin	0·24	0·53	0·37	0·38	−0·5
α_1	0·07	0·14	0·10	0·11	−0·5
α_2	1·07	0·07	0·58	0·57	+0·5
β	0·45	0·14	0·28	0·30	−1·2
γ	0·37	0·11	0·27	0·24	+1·9
			1·60	1·60	

In each case five 0·005 ml. portions of protein solution were applied to the paper.

Table 4. *Reproducibility of duplicate estimations at various dilutions of impure albumin*

Concentration of protein (g./100 ml.)	Optical density		Albumin (%)	β-Globulin (%)
	Albumin	β-Globulin		
1·1	1·423	0·139	91·0	9·0
	1·453	0·142	91·0	9·0
0·55	0·922	0·073	92·7	7·3
	0·817	0·078	91·3	8·7
0·275	0·317	0·020	94·0	6·0
	0·383	0·043	90·0	10·0

In each sample five 0·005 ml. portions were applied to the strip.

Dye binding capacities of proteins. It has been suggested (Kunkel & Tiselius, 1951) that the dye-binding capacities of the protein fractions differ. To investigate this, the dye intensity was compared with protein concentration, determined by biuret analysis, for albumin, γ-globulin and 'cold insoluble globulin' (fraction I of Cohn) (Morrison, Edsall & Miller, 1948). Fig. 6 shows that for equivalent amounts of albumin and fraction I, the density is directly proportional to protein concentration. In Table 1 the recoveries from mixtures of serum albumin and serum globulin are shown to be within 6 % of the expected values. Therefore for albumin, γ-globulin and fraction I, no differences in dye-binding capacity have been found.

Recovery experiments

Some examples of recovery experiments are shown. (i) With albumin added to serum (Table 2); (ii) with mixtures of serum and urine (Table 3).

Since this method was being used to compare the relative serum and urine protein concentrations in patients with proteinuria, it was necessary to determine the accuracy of recovery in these different fluids. Serum and urine proteins were therefore estimated separately and after mixture in various proportions. If any differences in binding capacity existed between the protein fractions, it should be apparent in these mixtures, since urine was relatively rich in albumin and serum relatively poor.

The errors in these estimations are closely comparable with the error in reproducibility of repeated estimations on the same serum, which was found to be ± 5 % of the total density on the paper (or of the total protein). There is thus an error approaching 100 % in the absolute estimation of a fraction comprising only 5 % of the total protein.

The reproducibility and sensitivity of the method is shown in the estimation of dilutions of a transfusion albumin preparation which contained about 10 % of a β-globulin (Table 4). By Tiselius U-tube estimation this preparation was 88 % albumin.

In this experiment a contaminating fraction was visible when a total of only 0·138 mg. protein was applied to the paper, and only 0·014 mg. of the impurity.

CONCLUSIONS

The evidence presented suggests that the method of filter-paper electrophoresis here described is quantitatively reliable when conditions are rigidly standardized. Many comparisons have been drawn between paper electrophoresis and 'classical' separation (Tiselius, 1937), some of which seem unsound in view of a number of fundamental differences in the two methods.

In the classical method the separation is carried out in buffer solution in a U-tube, and only partial separation of the components occurs, whereas in the paper method complete separation occurs (Fig. 2). The principle on which the concentrations of the various components are calculated also differs in the two methods.

The paper-strip method, as has been described, depends on dye-binding, which is directly proportional to relative protein concentrations under suitable conditions, and from the dye intensity the content of the various fractions can be calculated as a percentage of the total.

The U-tube method, on the other hand, depends on refractive index changes between the separated boundaries; this property is not specific for proteins, but depends also on lipid and carbohydrate in the protein components. For purified protein solutions the specific refractive increment can be determined, but in serum the composition of individual fractions is known to vary considerably (Longsworth, Shedlovsky & MacInnes, 1939) (e.g. the lipid content of the β-globulins in the nephrotic syndrome). Therefore estimation of protein concentrations of such sera when obtained by classical electrophoresis and based on nitrogen analysis may be considerably in error. The reproducibility and accuracy of the U-tube technique has been estimated at $\pm 1{\cdot}5$ % of the total protein (Armstrong, 1950). Evidence presented above suggests that the paper technique can give results to ± 6 % of the total protein present.

Thus, while the separation of pure proteins by electrophoresis on filter paper is not so accurate as is the 'classical' method, and it is not easily possible to measure absolute mobilities, yet it has quantitative advantages for mixtures of lipid and non-lipid containing proteins such as occur in serum, and moreover it is rapid, simple and relatively inexpensive to perform.

SUMMARY

1. A method is described for quantitative electrophoresis of proteins on filter paper, modified from that of Turba & Enenkel (1950).

2. A number of variables inherent in the method have been critically examined, including staining and washing of the papers; reproducibility of staining and optimum concentration of protein solutions; type of paper; 'tailing' of proteins on the paper during migration; dye-binding capacity of different protein fractions.

3. By recovery experiments and duplicate determinations, the quantitative accuracy of the method as described is found to be ± 6 % of the total protein present.

4. The comparative merits in the analysis of human sera of the paper method and the 'classical' Tiselius method are discussed. Apart from the

obvious advantages of simplicity and rapidity, electrophoresis on filter paper may be quantitatively more accurate in the analysis of pathological sera containing high proportions of lipid or carbohydrate.

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Time-Course of Injected Acetate in Normal and Depancreatized Dogs

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Acetic acid plays a very important role in intermediary metabolism both as degradation product of large molecules and as building block in numerous synthetic reactions.

It has been shown, mainly with the aid of isotopes, that acetic acid takes part in the syntheses of acetoacetate (Swendseid, Barnes, Hemingway & Nier, 1942), cholesterol (Bloch & Rittenberg, 1942), higher fatty acids (Rittenberg & Bloch, 1944, 1945), citrate (Stern & Ochoa, 1949), etc. Owing to the lack of specific chemical methods for the estimation of acetic acid, few studies of the metabolism of this compound have been made in which the amount of acetic acid metabolized was determined directly.

Recently Ciaranfi & Fonnesu (1952) have described a photometric micromethod for the estimation of acetic acid in blood and tissues and this procedure is suitable for such a type of investigation. With this method it has been shown that acetate is a normal constituent of blood and organs (Ciaranfi & Fonnesu, 1952), of lymph (Fonnesu & Ciaranfi, 1952) and of bile (Fonnesu, 1953*a*). Blood acetate originates either from the tissues or, by absorption, from the intestinal lumen. Skeletal muscle rapidly utilizes acetate conveyed by the blood, since the concentration of acetate in the arterial blood is greater than that in the veins draining the limbs (Fonnesu, 1951, 1953*a*; Fonnesu & Ciaranfi, 1952).

The concentration of acetate in the blood and tissues is relatively low probably because of the rapid turnover of the compound. Experiments with labelled acetate have shown that considerable amounts of acetate are transformed into other compounds and oxidized to carbon dioxide by the intact animal (Buchanan, Hastings & Nesbett, 1943; Lifson, Lorber, Sakami & Wood, 1948; Gould, Sinex, Rosenberg, Solomon & Hastings, 1949; Villee & Hastings, 1949; Pihl, Bloch & Anker, 1950; Hevesy, Ruyssen & Beeckmans, 1951; Coniglio, Anderson & Robinson, 1952; Hutchens & van Bruggen, 1952; van Bruggen, Claycomb, Hutchens & West, 1952). Significant quantities of acetate can also be oxidized by the eviscerated animal (Pihl *et al.* 1950; Wick & Drury, 1952). It has been observed that at least two extrahepatic tissues, that is, the heart (Pearson, Hastings & Bunting, 1949) and the diaphragm (Villee & Hastings, 1949) are capable of oxidizing acetate to carbon dioxide *in vitro*.

However, free acetate is known to be a metabolically inert substance and in order to react it requires activation. The work of Lynen & Reichert (1951) and of Lynen, Reichert & Rueff (1951) made it clear that 'active acetate' is in fact acetyl coenzyme A (Ac ~ CoA).

It is probable that free acetate is first converted into Ac ~ CoA. If this is true, the inhibition of such

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a reaction should lead to the accumulation of acetic acid. Since the concentration of acetate in the urine of human diabetics is greater than that in the urine of normal people (Caselli & Ciaranfi, 1946), it is reasonable to suppose that such a hyperacetaturia may be the consequence of a reduced acetate utilization by the diabetic body.

In the present work we describe experiments on the utilization of acetate by normal and depancreatized dogs, as indicated by the rate of disappearance of injected acetate from the blood stream.

MATERIAL AND METHODS

Normal adult dogs, weighing between 15 and 20 kg., were used. Pancreatectomy was performed by the technique of Haberland (1934) under Pentothal:ether anaesthesia. The depancreatized animals were subjected to the experiment not less than 2 or 3 days after the removal of the pancreas and during this time received neither insulin nor any other treatment. All the animals were fasted for 12 hr. before the experiment.

Normal and depancreatized dogs were given an intracardiac injection of sodium acetate:acetic acid mixture, pH 7·4, containing 25% (w/v) of acetic acid. The amounts injected, expressed as acetic acid, were as follows: 50, 100, 150, 250 mg./kg. body weight. The injection time was always 1 min. Before and at various intervals after the injection, arterial blood samples were taken and the following compounds estimated: acetic acid (Ciaranfi & Fonnesu, 1952), ketone bodies (Weichselbaum & Somogyi, 1941), glucose (Folin & Wu, 1919), cholesterol (Bloor, 1916) and serum phosphate (Fiske & Subbarow, 1925). At the time of the acetic acid estimation, the coloured samples were centrifuged before the photometric reading. A Coleman Spectrophotometer, model S. 14, was used.

Immediately before the acetate was injected, the bladder was emptied by catheter; 30 min. after the injection the bladder was again emptied. The acetic acid concentration in both samples of urine was then determined after Fonnesu (1953*b*). Glucose was estimated in the urine by the Fehling reagent (Bertho & Grassmann, 1936).

RESULTS

Table 1 gives representative values for glucose, ketone bodies and acetate in the blood and for glucose in the urine of both normal and depancreatized dogs before the acetate injection. It will be seen that the blood acetate level is higher in the diabetic than in the normal dogs.

Fig. 1 shows the rates at which different amounts of acetate are removed from the blood of normal and

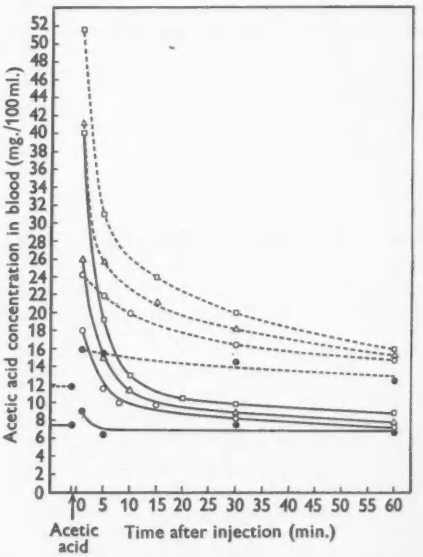

Fig. 1. Time-course of injected acetate in normal (——) and depancreatized (- - -) dogs. The amounts injected, expressed as mg. acetic acid/kg. body weight, were: 50 (●); 100 (○); 150 (△); 250 (□). Each point represents the average of at least three experiments.

Table 1. *Experimental data for normal and depancreatized dogs before the acetate injection*

Type of animal	Arterial blood: Glucose (mg./100 ml.)	Arterial blood: Total ketone bodies (expressed as mg. β-hydroxybutyric acid/100 ml.)	Arterial blood: Acetic acid (mg./100 ml.)	Urine: Glucose excreted (g./l.)
Normal				
Mean ± S.E.	108 ± 2·05	0·94 ± 0·07	7·0 ± 0·41	0
No. of observations	7	7	7	7
Range	100–112	0·69–1·21	5·0–8·0	0·0–0·0
Depancreatized				
Mean ± S.E.	340 ± 24·48	10·98 ± 1·00	11·2 ± 1·18	28·8 ± 4·36
No. of observations	6	6	6	6
Range	280–435	7·9–13·8	8·2–15·4	12–40

Table 2. *Acetic acid concentration in urine (mg./100 ml.) of normal and depancreatized dogs before acetate injection*

	Normal	Depancreatized
Mean ± S.E.	4·6 ± 0·92	9·8 ± 0·62
No. of observations	6	4
Range	2·8–8·0	8·5–11·3

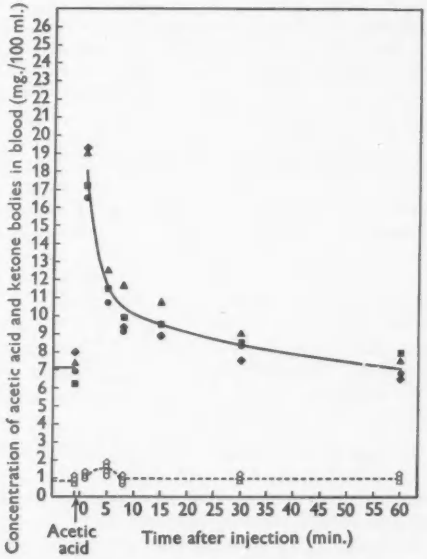

Fig. 2. Relationship between the acetate-disappearance (——) and the formation of ketone bodies (- - - -) in normal dogs. The amount of acetic acid injected was 100 mg./kg. body weight. Points represent values for single animals.

depancreatized dogs. Injected acetate disappears very rapidly from the blood at a rate which is related to the initial blood concentration. Acetate disappears more rapidly from the blood of normal dogs than from the blood of diabetic animals. Taking the half-time as an index of the phenomenon it is observed that in the diabetic animals such a value is 2–6 times greater than normal.

Table 2 shows that the concentration of acetic acid in the urine of diabetic dogs is higher than that in normal animals even before acetate injection.

It should be noted that 30 min. after the injection of acetate the amount of urine found in the bladder, which had been emptied before the injection, was only a few ml. Since the concentration of acetate in these samples was slightly higher than that found in the urine before the injection, the amount of injected acetate eliminated by way of the kidneys (0·2–3 % of the amount injected) may be neglected.

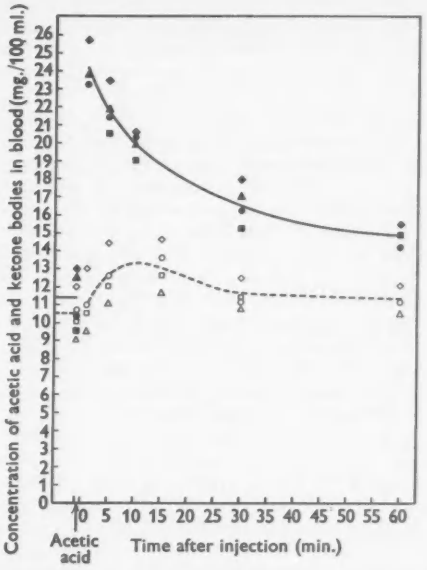

Fig. 3. Relationship between the acetate-disappearance (——) and the formation of ketone bodies (- - - -) in depancreatized dogs. The amount of acetic acid injected was 100 mg./kg. body weight. Points represent values for single animals.

Experiments were next carried out in which the concentrations of both acetate and ketone bodies in blood were measured at suitable time intervals following an injection of acetate. Fig. 2 shows the results obtained with normal dogs and Fig. 3 with diabetic dogs, respectively. It is evident that in normal dogs the blood ketone level rises at the time corresponding to the maximal drop of blood acetate and very rapidly returns to the initial values (Fig. 2). In the diabetic dogs the concentration of ketone bodies in the blood, much higher than normal even before acetate injection, increases much more than the normal and tends to return to the initial values more slowly (Fig. 3).

No significant change following the injection of acetate has ever been observed in the concentrations of glucose, phosphate and cholesterol in the blood of both the normal and depancreatized dogs.

DISCUSSION

The data show that acetate, injected into the blood stream, disappears very rapidly and that the amount of the injected acetate eliminated by the urine is very small. Although it is possible that a part of the injected acetate is secreted with the

various digestive juices, such a possibility does not explain the rapid disappearance of acetate from the blood, since the acetate is rapidly absorbed from the intestine, especially by the lymphatic system (Fonnesu & Ciaranfi, 1952; Fonnesu, 1953*a*). It may be concluded that the injected acetate is rapidly metabolized by the tissues. This conclusion is in agreement with the results of analogous experiments with labelled acetate, in which the acetate utilization was determined by the rate of incorporation of ^{14}C in those compounds of which acetate is the precursor. The concentration of acetate in the blood of diabetic dogs is greater than that of normal dogs, and acetate is utilized more slowly in the former than in the latter. The acetic acid concentration in the urine is also increased in the diabetic dog, this being in agreement with previous observations on the urine of human diabetics (Caselli & Ciaranfi, 1946).

These results may be interpreted in a number of ways, viz. in the diabetic animal (*a*) the level of CoA is lowered, (*b*) the acetylation of CoA is reduced, (*c*) the utilization of Ac~CoA is impaired, or (*d*) more than one of the above-mentioned mechanisms are involved. These hypotheses can be discussed in the light of previous data found in the literature. A reduced synthesis of CoA in diabetes, although not demonstrated, appears probable if it is assumed that the synthesis of this substance requires high-energy phosphate bonds, the production of which is lowered in diabetes because of impaired glucose oxidation (Kaplan & Greenberg, 1944; Kaplan, Franks & Friedgood, 1945; Cutolo & Siliprandi, 1952). The reduced synthesis of CoA could also be due to a diminished availability of its precursors, and it is known that in diabetes the amounts of SH compounds are reduced (Lazarow, 1949). On the other hand, the acetylation of CoA could be diminished in diabetes since the formation of Ac~CoA requires adenosine triphosphate (ATP) (Lipmann, 1945). Charalampous & Hegsted (1949*a*, *b*) have shown that in the alloxan-diabetic rat the acetylation of *p*-aminobenzoic acid is markedly reduced and that acetylation is restored to normal values by the injection of ATP, acetylphosphate, and some di- and tri-carboxylic acids of Krebs's cycle, the most active of which is malate. Insulin also reactivates, although more slowly, the acetylation processes in diabetes. None of these substances influences the rate of acetylation in the normal rat. These observations support the hypothesis that in diabetes the amount of Ac~CoA is reduced because of the reduction of oxidative phosphorylation necessary for the synthesis of CoA and for its acetylation with acetate.

The stages after Ac~CoA have been more extensively studied in diabetes. It is well known that the synthesis of higher fatty acids is markedly reduced (Brady & Gurin, 1950), that the coupling with oxalacetate must be decreased because of oxalacetate deficiency (Frohman, Orten & Smith, 1951) and that the acetylation processes are greatly diminished (Charalampous & Hegsted, 1949*a*, *b*). On the other hand, the cholesterol synthesis, which needs CoA (Klein & Lipmann, 1953), is not lowered in diabetes as compared with the normal (Brady & Gurin, 1950), whilst the level of ketone bodies, the synthesis of which requires CoA (Stadtman, Doudoroff & Lipmann, 1951) is greater in diabetic than in normal animals. It is to be noted here that our results demonstrate that the injection of acetate leads, in the diabetic animal, to a higher and more prolonged increase in the blood ketone level as compared with the normal. It is probable, however, that the high level of ketones does not depend so much upon the increased synthesis of the just mentioned substances but upon the reduced ketolysis.

It may be concluded from the above data that the reduced acetate utilization in the diabetic dog depends on many factors, among which the most important probably is the reduced formation of Ac~CoA. Experiments to clarify this point are still in progress.

SUMMARY

1. Acetate utilization, both in normal and depancreatized dogs, has been studied *in vivo* by following the rate of disappearance of acetate injected directly into the blood stream.

2. Acetic acid concentration, both in the blood and in the urine of diabetic animals, was found to be higher than normal even before acetate injection.

3. Injected acetate always disappeared from the blood very rapidly with a rate which depended upon the initial blood concentration of the compound.

4. The amount of injected acetate eliminated by way of the kidneys was small.

5. The rate of disappearance of acetate from the blood, was markedly reduced in diabetes.

6. Acetate injection produced in diabetic animals a higher and more prolonged increase in the concentration of blood ketone bodies as compared with the normal. The highest blood concentration of ketone bodies was observed at a time corresponding to the maximal drop of blood acetate.

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